

Division of

Cancer Etiology

1986 Annual Report
Volume II

October 1, 1985-
September 30, 1986

U.S. DEPARTMENT
OF HEALTH
AND HUMAN SERVICES

National
Institutes of
Health

National
Cancer
Institute

Bethesda,
Maryland 20892

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ANNUAL REPORT OF
THE LABORATORY OF BIOLOGY
CHEMICAL AND PHYSICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1985 through September 30, 1986

The Laboratory of Biology plans, develops, and conducts *in vitro* and *in vivo* investigations aimed at elucidating the role of chemical, physical and biological agents in the modulation of carcinogenesis. Coordinated biochemical and biological studies utilizing human and animal cell models are used to characterize the cellular alterations associated with carcinogenesis. These include: assessment of the effect of physiologic host mediating factors, determination of cell surface changes, evaluation of the relationships between DNA metabolism, chromosome alterations, and carcinogenesis.

In general terms, cancer is considered a genetic disease that occurs as a result of genomic alterations in somatic cells. The process of carcinogenesis induced spontaneously or experimentally involves carcinogenic agents, target cells, and the interaction of the host and transformed cells. The primary objective of the Laboratory of Biology is the determination of the mechanism of "competence" that makes the cell susceptible to transformation. Thus, it will become possible to determine how transformability differs from nontransformability and how the transformation may be modified. The two sections of the Laboratory of Biology complement each other in this endeavor. The Somatic Cell Genetics Section is concerned with the relevant changes in chromosomes and DNA metabolism which regulate gene expression responsible for neoplastic transformation. By superimposing molecular events on biological observations, conclusions concerning gene expression relevant to control of differentiation and cancer will also be possible. The Tumor Biology Section emphasizes host interactions, particularly from an immunological point of view. The objective of these studies is to examine phenotypic changes at the cell surface to identify the effectors that prevent or modulate transformation and to study their mode of action.

Animal cells transformed by diverse classes of carcinogens, including environmental agents incriminated on the basis of epidemiologic studies are characterized by a combination of common but not universal changes: altered morphology, motility, chromosome changes, and indefinite growth. Human cells differ from animal cells in that they have increased resistance to transformation by any of the well-known inducing agents: X-rays, ultraviolet rays, chemicals, and viruses. Furthermore, human cells transfected in culture with transforming genes are also more resistant than are animal cells to transformation by these genes. By utilizing protocols developed with animal cells on normal human fibroblasts, transformation of normal human fibroblasts by known carcinogens as reflected by growth in agar, extended life span, and invasion of the subcortical brain regions of nude mice resulted in death of the animals. The ultimate objective, to develop a reproducible model in which human cells are converted to permanent malignant cell lines in a predictable fashion, has not yet been achieved. Therefore, mechanism(s) controlling malignancy must be more stringent for human cells than for animal cells used in standard *in vitro* assays.

Currently both human fibroblasts and epithelial cells are being studied. Although only epithelial cells exhibit continuous but regulated stem cell cycling *in vivo*,

both are capable of multiplication *in vitro*. Furthermore, mesenchymal and epithelial cancers occur in humans. Human foreskin is used as the predominant source of cells. The use of epithelial cells has required the development of a new medium, MCDB153-LB, that maintains the nondifferentiated keratinocyte cells and that allows cell division to occur approximately every 24 hrs. As with the other serum-free media for growth of human epithelial cells, MCDB153-LB medium is a modification of one originated by R. Ham (University of Colorado). The ingredients in the medium are completely defined except for the bovine pituitary extract.

A multi-pronged cocarcinogenesis approach is being utilized to overcome the genome stability and genetic suppression present in human cells. Papillomaviruses, known etiologic agents that cause benign proliferation of skin and mucosa, result in tumors that are histologically classified as papillomas or fibropapillomas. Because HPV16 presence is also associated with human carcinomas, particularly genital cancer, and their tumor derived cell lines, HPV 16 DNA has been transfected into human foreskin fibroblasts or epithelial cells to develop a model for studying cocarcinogenesis. The transfected cells are also useful for elucidating the molecular biology of HPV 16. A recombinant HPV 16 DNA (containing a head-to-tail dimer of the full length HPV 16 genome and a selectable marker, G418, that induces tumorigenic transformation of NIH/3T3 cells) was used to transfect foreskin-derived fibroblasts and keratinocytes by the calcium phosphate precipitation method. After G418 selection a cell population was obtained which had an increased saturation density and extended life span that is currently greater than 100 population doublings (PD) compared to the control which senesced at about 50 doublings. Foci of piled-up cells formed in confluent cultures. Anchorage-independent growth in agarose was observable from PD 5 post-selection. The frequency of anchorage-independent colonies progressively increased with time in culture. Furthermore, X-irradiation (400R) of the transfected cells at PD 7 markedly accelerated morphologic transformation. Foci formation appeared 2 PD post X-irradiation; foci-derived cells were anchorage independent (2%). Nontransfected X-rayed cells fail to form foci and senesced as did controls. Southern blot analysis of transfected cells (d0) and cells derived from an X-ray-induced focus (dX) showed the presence of HPV 16 sequences in multiple copies. Furthermore, both transformed d0 and dX express several HPV 16 mRNA species. Similarly, keratinocytes with an indefinite life span that possess HPV 16 and RNA expression have been developed. The keratinocytes are maintaining their nondifferentiated character *in vitro*. Thus, HPV 16-transfected human fibroblasts and keratinocytes provide a suitable model for studying molecular biology of HPV 16 and for cocarcinogenesis.

A model for analyzing the function of HPV 16 was demonstrated by two-stage transformation of NIH/3T3 cells by transfection with a recombinant HPV 16 DNA the full length of HPV 16 and the *neo* gene. Expression of transforming activity was unique because of the long latency period required for induction of morphologic transformation. Cells were selected for resistance to G418 (PM3T3G) or foci formation without G418 selection (PM3T3Fo). Different morphologies were noted in PM3T3Fo and PM3T3G lines in confluent cultures; cell densities averaged two- to threefold higher in PM3T3G and five- to sevenfold in PM3T3Fo higher than in control NIH/3T3 confluent cultures. All HPV 16-transfected lines had a reduced serum requirement for growth. Tumorigenic latency in nu/nu mice was one week for Fo and greater than 14 weeks for G lines. Some G lines showed progressive transformation. Both G and Fo lines had extrachromosomal and rearranged HPV 16 DNA but the proportion of the integrated form is significantly greater in Fo lines. E6 mRNA was the major transcript in all lines. Moreover, tenfold more HPV 16-specific mRNA was detected in G compared to Fo cells with a consistent set of qualitative and relative quantitative differences. This differential gene expression may be reflected in the physical state of HPV 16

DNA. Moreover, levels of HPV 16 gene expression are insufficient for a tumorigenic phenotype. However, a primary effect of HPV 16 is considered to be growth modulation. These results suggest that a specific interaction between HPV 16 genome and some cellular factors is required for progression toward malignant transformation.

Chromosome changes are implicated in certain human cancers. In fact, most cancers are characterized by chromosome markers. The breakpoints in various rearrangements involve a limited number of chromosome sites and frequently occur at the sites of cellular proto-oncogenes. With chromosome rearrangements, proto-oncogenes can be transposed to new sites near enhancing or promoter sequences resulting in changes in proto-oncogene regulation. The chromosome alterations in solid tumors, are usually very complex compared to hematological malignancies. This complexity may obscure primary changes actively involved in the process of neoplastic development.

The increased incidence of malignant mesothelioma of the lung is associated with asbestos exposure. Therefore, chromosome analysis of mesotheliomas is relevant to characterizing this cancer. In an analysis of nine cases, all of which were examined prior to therapy, eight have a common chromosome alteration. The abnormality consisted of either interstitial or terminal deletions involving the long arm of chromosome 3. Interstitial deletions were observed in two cases: a deletion from the normally metacentric chromosome 3 to form a submetacentric and an inserted segment the size of a large band at 3p14-21 that restored the short arm, long arm ratio. In the six other cases, the deletion was terminal. The common abnormality at 3p14-21 may be critical to development of neoplasia. Specific alterations of the short arm of chromosome 3 (3p13-24) have also been detected in familial renal carcinoma, small cell carcinoma of the lung, mixed parotid gland tumors, B-prolymphocytic leukemia, ovarian carcinoma, malignant lymphomas, and rhabdomyosarcoma. The involvement of this specific region in such diverse malignancies may be due to the presence of a fragile site at 3p14-21, which is the most common in the human genome. At present no proto-oncogene is known to be located at 3p14-21. The possibility is raised that the 3p region may contain one or more "suppressor" or "regulatory" genes which could be either lost or inactivated.

Among the best studied human tumor lines is HeLa, a cervical carcinoma, whose karyotype has been virtually unchanged since it was established in culture in 1952. Because HPV 18 sequences are integrated into the genome, the chromosome localization of these viral nucleic acid sequences was determined. This information was desirable to understanding the interaction of DNA viral material with the cellular genome. Four main labeling sites of HPV 18 sequences were found to be integrated; three of these also involved the loci of *c-myc*, *c-abl*, and *c-sis*. Furthermore, the possibility exists that integration may have been facilitated by the coincidence of fragile sites at these loci. Thus, the interaction of HPV 18 with the cellular genome appears relevant to the development of cervical cancer.

The observation that HPV16 DNA sequences are integrated in HeLa cell chromosomes at fragile sites and/or sites of proto-oncogenes rather than at random sites may indicate that integration at a specific locus is critical to conversion to malignancy. In general, HPV18 is integrated into the host genome in cervical tumor cell lines. The transition to tumorigenicity associated with the integration and altered expression of HPV 16 DNA into the host genome of NIH/3T3 cells also supports the importance of a specific host-cell viral interaction. The transfected human fibroblast

or epithelial cells mimic the nonmalignant NIH/3T3 cells. It is predicted that further insults which cause integration of HPV 16 sequences will be pivotal to progression to the malignant state.

There is a need for additional experimental models to study the series of steps initiated by damage to DNA which leads to the genomic changes that result in malignancy. The guinea pig model, with its discrete preneoplastic stages, provides an ideal opportunity for studying multistage oncogene-induced neoplasia. Five independent tumorigenic guinea pig cell lines initiated by diverse chemical carcinogens (aromatic aryl hydrocarbons or alkylating agents) contain activated oncogenes that transform NIH/3T3 cells. Oncogene activation occurred at a late stage of carcinogenesis closely associated with acquisition of tumorigenicity. Sequence analysis of the cloned oncogene of one line shows that it is an *N-ras* gene activated by an AT to TA transversion at the third position of codon 61. This results in the insertion of histidine instead of glutamine. The other four lines contain the same activated *N-ras* gene with the identical mutation. These results suggest that the mutational event was independent of the mutagenic activity of the initiating carcinogen. The activation of *ras* genes in *in vitro* models usually occurs in an early step and the specific activating mutation is generally related to the initiating carcinogen. The contrasting results with the guinea pig model demonstrates that *ras* activation need not occur at a specific interval in the carcinogenesis process.

Lymphokines are now recognized as important regulators of cell growth and differentiation. As such they have the potential to interact with and to modify many stages in the development of neoplasia. One lymphokine, leukoregulin, which was first identified within this Laboratory, has anticarcinogenic and antitumorigenic properties. The uniqueness of this hormone is that it acts directly on the target cell in contrast to stimulating the activity of cytotoxic effector cells as do lymphokines like interferon and interleukin-2. Interaction of leukoregulin with the target cell is followed by alterations in cell surface conformation, plasma membrane integrity, cell surface glycoprotein expression, and DNA synthesis. The ability of leukoregulin to effect changes in both cell metabolism and structure makes this "biological" a valuable probe for investigating the extragenetic control of carcinogenesis and the neoplastic growth of transformed cells.

Investigations into the molecular pathways of the anti-carcinogenic and tumor proliferation inhibitory action of leukoregulin have continued during this past year to focus upon the biochemical events responsible for destabilizing the target cell membrane in preneoplastic and in neoplastic cells. A major function of the plasma membrane is to maintain the ion flux necessary to support the state of differentiation and function of the cell. The mechanisms underlying the perturbation in plasma membrane permeability following target cell interaction with leukoregulin have, therefore, been studied by contrasting the changes in leukoregulin-treated cells with those after exposure of target cells to a variety of membrane-active agents affecting monovalent and divalent ion transport. Tumor and other abnormal cells are more than 10,000-fold more sensitive to the proliferation inhibitory action of leukoregulin than are normal cells. The reason for the differential sensitivity remains to be defined and is important because changes in calcium flux appear to be of major importance in many cytotoxic and immunoregulatory actions and in being able to modulate carcinogenesis. Elucidation of the molecular events by which leukoregulin affects calcium flux may thus provide fundamental new insights into our understanding of immunoregulation and into how manipulation of normal physiological processes contribute to or may be used to prevent the development of neoplastic transformation.

Compounds including ouabain, amphotericin B, calcium ionophores A23187, X-537A and ionomycin, phospholipase C and A2, and phytohemagglutinin which increase intracellular calcium levels mimic the changes in membrane conformation and permeability induced by leukoregulin. Calmodulin, calcium channel blockers and both sodium and potassium ionophores and channel blockers exhibit no flow cytometrically detectable target cell membrane destabilizing activity. The calcium modulators are active over a wide range from 10^{-3} to 10^{-10} M and the one paralleling the kinetic activity of leukoregulin most closely is the calcium ionophore A23187. The ability of compounds that increase intracellular ionic calcium to increase membrane permeability suggests that leukoregulin may exert its action by increasing intracellular calcium which, in turn, increases membrane permeability. The ability of calcium ionophores which transport calcium across the membrane, but not calcium membrane-channel blockers, to affect membrane permeability indicates that if leukoregulin acts by increasing intracellular calcium levels, it does so using a mechanism distinct from calcium channel transmembrane transport.

Intracellular ionic calcium is an important ion in the maintenance of membrane potential and in a variety of pathways regulating cell growth and function. Flow cytometric measurements of intracellular ionic calcium in leukoregulin-treated human K562 leukemia cells using the calcium binding fluorescent probes quin2 and, more recently, indo-1 reveal that leukoregulin induces a very large transient increase in intracellular calcium that may reach a level 1,000 times higher than the basal level from one to ten minutes after cell exposure to the lymphokine. Treatment of the cells with calcium ionophore A23187 produces a more immediate transient increase in intracellular calcium within seconds of ionophore exposure followed by a second transient increase several minutes later indicating that although leukoregulin may increase intracellular calcium levels, it does not function solely as an ionophore. The increase in membrane permeability and rise in intracellular calcium following target cell exposure to calcium ionophore is completely dependent upon the presence of extracellular calcium. The changes induced by leukoregulin, in contrast, are relatively independent of extracellular calcium, suggesting that the leukoregulin-induced increase in calcium flux is due to release of bound calcium from intracellular stores such as those located in the endoplasmic reticulum as opposed to the transmembrane influx of extracellular calcium. The rise in intracellular ionic calcium minutes after interaction of the cell surface with leukoregulin is consistent with leukoregulin binding to its cell surface receptor leading to stimulation of the inositol phospholipid signaling system or a modulation of the calcium binding protein, calmodulin, with a resulting increase in intracellular calcium. The immediate and continuous opening and closing of plasma membrane monovalent but not divalent ion channels are also observed by electrophysiological patch clamp measurements of the membrane potential of K562 cells in the presence of leukoregulin providing additional evidence that the increased calcium flux results from intracellular mobilization of bound calcium.

Interaction of leukoregulin with the target cell initiates a series of events commencing with an immediate, continuous opening and closing of plasma membrane monovalent ion channels, a marked increase within a minute or so in intracellular ionic calcium and several minutes later, a progressive increase in plasma membrane permeability followed by increased sensitivity of the affected target cell to natural killer lymphocyte cytotoxicity accompanied by inhibition of target cell DNA synthesis. Leukoregulin's alteration of calcium flux and membrane permeability without causing cytolysis demonstrates that neither event alone or in combination is necessarily cytolethal. This is consistent with leukoregulin serving as an

immunologic hormone whose function is to condition abnormal cells for eventual elimination by the host. Modulation of cell surface conformation, membrane fluidity, and permeability to the same degree present in natural lymphocyte-mediated cytotoxicity reactions signifies that leukoregulin, through transmembrane signaling, has the potential to control the specificity of membrane cytotoxicity by decreasing the proliferative rate of the target cell and increasing the vulnerability of its plasma membrane to additional cytoregulatory agents. Changes in calcium flux may also be important in the anticarcinogenic action of leukoregulin but diverge from the molecular pathway leading to increased membrane permeability since leukoregulin does not decrease membrane integrity nor inhibit DNA synthesis in non-transformed cells serving as targets for carcinogenesis.

Neoplastic cells in all phases of the cell cycle are sensitive to the proliferation inhibitory action of leukoregulin. Flow cytometric analysis of human K562 leukemia cells treated with increasing concentrations of leukoregulin to inhibit more than 90 percent of the cell replication demonstrates that the percentage of cells in each phase of the cell cycle remains constant during 72 hours of leukoregulin treatment. Fluorescence-activated cell sorting of leukoregulin plasma membrane permeability altered cells also reveals that cells in each phase of the cell cycle are susceptible to the membrane perturbing action of leukoregulin. Flow cytometric analysis of the sorted cells indicates that cells in the "S" phase of the cell cycle may be more sensitive to the acute membrane destabilizing action of leukoregulin. This cell cycle independent pattern of inhibition of cell replication and its rapid reversibility are quite different from the cell cycle phase-specific blocks observed with the typical inhibitors of cell replication affecting protein and/or nucleic acid synthesis. These observations, if they prove to be generally applicable to tumor or other abnormal cells, are potentially of great usefulness both in terms of understanding the control of cell kinetics as well as in developing agents that are able to inhibit the proliferation of and to destabilize the plasma membrane of cells independently of their position in the cell cycle. This would be particularly valuable in the control of tumor and other abnormal cells that are present in the nonreplicating phases of the cell cycle, stages in cell growth that are difficult to selectively control with anti-proliferative agents.

Leukoregulin is a unique and potent new "biological" for use in investigations into the cause and control of cancer. It is the first lymphokine with the recognized ability to directly interact with the target cell to prevent radiation and chemical carcinogenesis as well as being able to modulate the development of transformation at several stages in the transition to neoplasia. In addition it is the only lymphokine known to also increase the susceptibility of pre-neoplastic and neoplastic cells to destruction by natural killer lymphocyte cytotoxicity and to reversibly inhibit DNA synthesis in the cells. The specificity of the latter two actions is extremely high and is mediated through leukoregulin-induced destabilizations of the cell surface and in the integrity of the target cell plasma membrane. In this manner leukoregulin may function as one of the earliest intrinsic mediators in natural lymphocyte cytotoxicity by controlling the specificity and direction of the immunoregulatory action directly at the surface of the pre-neoplastic or neoplastic target cell. The specificity and direct target cell actions of the lymphokine make leukoregulin a valuable new probe for study of the extragenetic prevention and control of carcinogenesis and as a potential agent for the control of tumor and other abnormal cell growth.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP04629-21 LB
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Regulation of Stages of Carcinogenesis Induced by Chemical or Physical Agents		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: J. A. DiPaolo Chief LB NCI		
Others: S. Amsbaugh Microbiologist LB NCI J. Doniger Senior Staff Fellow LB NCI L. Pirisi Visiting Fellow LB NCI N. C. Popescu Microbiologist LB NCI S. Yasumoto Visiting Associate LB NCI		
COOPERATING UNITS (if any) none		
LAB/BRANCH Laboratory of Biology		
SECTION Somatic Cell Genetics Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 6	PROFESSIONAL: 5.5	OTHER: .5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) To understand the nature of interrelationships between carcinogenesis and DNA metabolism, chromosome structure, and biological reagents, <u>in vivo</u> and <u>in vitro</u> approaches are being used in model systems. Evidence has been accumulated that human cells are not transformed to the malignant state by chemical, physical, or viral carcinogenic agents that are effective with animal cells. Therefore, a multifaceted cocarcinogenesis approach is being used with human cells to overcome the genome stability or genetic suppression of transformation. Human fibroblasts and prokeratinocytes derived from foreskin have been transfected with recombinant HPV 16 DNA. Stable, intact extrachromosomal HPV 16 sequences have been demonstrated in transfected cells which acquire an indefinite life span and anchorage independent growth. Studies with cervical carcinomas and NIH/3T3 transfected cells have shown the importance of integration of papilloma sequences for tumorigenicity. In HeLa cells four chromosome sites contain HPV 18 sequences. Three of these are also associated with fragile sites. Moreover, this is the first demonstration of integration of a human DNA virus near sites of proto-oncogenes. Two stages of transformation were observed in HPV 16-transfected NIH/3T3 cells. Transfected cells have a higher proportion of integrated HPV DNA and decreased HPV 16 expression when the cells become tumorigenic. A common chromosome change associated with a common chromosome fragile site was found in eight of nine malignant mesotheliomas derived from asbestos exposed patients. Because no proto-oncogene has been identified at this location, an important suppressor or regulatory gene may have been lost or inactivated. Preneoplastic and tumorigenic guinea pig cells have been analyzed for oncogenes. Activation of N-ras is associated with acquisition of tumorigenicity. The identical AT to TA transversion was found in N-ras in five tumorigenic lines initiated by various carcinogens. This mutation was not induced by the mutagenic activity of the initiating carcinogen.		

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Joseph A. DiPaolo	Chief	LB	NCI
S. Amsbaugh	Microbiologist	LB	NCI
J. Doniger	Senior Staff Fellow	LB	NCI
L.A. Pirisi	Visiting Fellow	LB	NCI
N.C. Popescu	Microbiologist	LB	NCI
S. Yasumoto	Visiting Associate	LB	NCI

Objectives:

The overall approach to problems in carcinogenesis is to investigate factors and mechanisms responsible for the modulation of neoplastic transformation of human and other cells, vital aspects in the etiology and prevention of cancer. The specific objectives are (1) to define the role of chemical, physical, and biological agents pertinent to the process of carcinogenesis; (2) to characterize the cellular and chromosomal alterations associated with carcinogenesis; (3) to evaluate the relationships between DNA metabolism and carcinogenesis.

Methods Employed:

All procedures are performed with the view of understanding the underlying mechanism controlling the progression of the transformation phenomenon. Such an approach is required to determine whether the observed transformation is due to the direct or indirect effect of the carcinogen and to study the events associated with neoplastic transformation. Cultures are made with freshly isolated cells from animals and humans as well as cell lines which exhibit some of the properties associated with nontransformed cells. Cells derived from whole embryos or specific organs are grown in medium with or without serum in the presence or absence of irradiated cells (feeder cells) and exposed to carcinogen transplacentally or prior to or subsequent to seeding the cells in plastic dishes. To maintain protracted logarithmic multiplication of human prokeratinocytes, a complete medium MCDB 153-LB was devised consisting of variable concentrations of amino acids and hormones but without serum. This medium allows the use of differentiation markers in conjunction with the transformation process. Transfection of mammalian cells is accomplished by calcium phosphate precipitation, DEAE-dextran, protoplast fusion or electrofusion. Analysis of DNA and RNA in transformed cells is by agarose gel electrophoresis, Southern blotting, DNA restriction analysis, gene cloning, c-DNA cloning, and RNA and DNA sequencing. High resolution banding methods of prophase and prometaphase are used to identify structural chromosome alterations. Chromosome changes are further characterized by specific methods for visualization of constitutive heterochromatin (C band), ribosomal genes (N-band) and immunochemical methods using anti-nucleoside antibodies on denatured chromosomes. The molecular *in situ* chromosome hybridization technique devised by us is being used for gene mapping and proto-oncogenes transposition on cancer cells.

Major Findings:

Human cells differ from animal cells in that they are resistant to neoplastic transformation by any of the well-known inducing agents: X-rays, ultraviolet rays, chemicals, and viruses. Consequently, a multifaceted cocarcinogenesis approach is being utilized to overcome genome stability and genetic suppression of transformation associated with human cells. Because human papillomaviruses (HPV), known etiological agents of benign proliferation of skin and mucosa, are histologically classified as papillomas or fibropapillomas and because their presence is associated with human carcinomas, HPV DNA has been transfected into human foreskin fibroblasts and epithelial cells. To facilitate the understanding of the role of HPV in transformation, molecular aspects of the HPV 16-transfected NIH/3T3 transformation model have also been studied. Human fibroblasts and keratinocytes transfected with recombinant HPV 16 DNA exhibit an extended lifespan relative to their respective controls. After neomycin selection, fibroblasts exhibit increased saturation density and indefinite lifespan (> 120 population doublings). Foci of piled-up cells formed in confluent cultures and anchorage independent growth was observable; the frequency progressively increased. Moreover, X-irradiation markedly accelerated transformation. X-irradiated nontransfected cells failed to form foci and senesced as did controls. Southern blot analysis of transfected cells (d0) and cells derived from an X-ray-induced focus (dX) showed stable intact HPV 16 sequences in multiple copies. Furthermore, both d0 and dX express several HPV 16 mRNA species. Transfected keratinocytes with an indefinite life span also possess and express HPV 16 DNA.

Two-stage transformation occurred in NIH/3T3 cells transfected with recombinant DNA containing full length HPV 16 and the *neo* gene. Transfected cells were either selected for resistance to G418 (PM3T3G) or foci formation without G418 selection (PM3T3Fo). Different morphologies were noted in PM3T3Fo and PM3T3G lines in confluent cultures; cell densities averaged two- to threefold in PM3T3G and five- to sevenfold in PM3T3Fo higher than in control NIH/3T3 confluent cultures. All HPV 16-transfected lines had a reduced serum requirement for growth. Tumorigenic latency in nu/nu mice was 1 week for Fo and greater than 14 weeks for G lines. Some G lines showed progressive transformation in that foci eventually appeared. Both G and Fo lines had extrachromosomal and rearranged HPV 16 DNA but the proportion of the integrated form is significantly greater in Fo lines. E6 mRNA was the major transcript in all lines. Moreover, tenfold more HPV 16-specific mRNA was detected in G compared to Fo cells with a consistent set of qualitative and relative quantitative differences. This differential gene expression may be reflected in the physical state of HPV 16 DNA. Moreover, levels of HPV 16 gene expression are not sufficient for a tumorigenic phenotype. However, a primary effect of HPV 16 is considered to be growth modulation. These results suggest that a specific interaction between HPV 16 genome and some cellular factors is required for progression toward malignant transformation.

In most cases of human genital cancer, HPV 16 or 18 sequences are integrated into the host genome. The localization of these viral nucleic acid sequences at specific genomic sites is important to the understanding of the interaction of virus with the cellular genome. *In situ* hybridization was used to assign the integration sites of HPV 18 in HeLa, a cervical carcinoma cell line with 10-50 copies of HPV 18. Two

major sites were identified: 8q23-24, which contained 26% of the grains and coincided with c-myc location; 5p11-13 on an abnormal chromosome 5 had 9% of the observed grains. Two other sites at 9q31-34 and at 22q12-13 on an abnormal chromosome 22 include the locations of c-abl and c-sis genes, respectively. This is the first demonstration of integration of a human DNA virus near sites of proto-oncogenes. The coincidental location of HPV 18 DNA sequences with myc may have special significance because myc expression is amplified in cervical cancers.

Malignant mesothelioma (MM) is a neoplasia causally associated with asbestos exposure. The chromosome constitution of nine MMs derived from asbestos-exposed patients prior to therapy was determined. One tumor had a normal karyotype, and the others were near diploid or triploid with chromosomally abnormal clones and variable numbers of abnormal chromosomes ranging from 1 to 15. A specific alteration of 3p14-21 consisting of either interstitial or terminal deletions was identified in eight cases. The abnormality at 3p14-21 may be critical to the neoplastic development of mesothelioma. Significantly, the only case without a 3p defect was from a patient who had a complete response to chemotherapy and is still in remission. Breakage susceptibility at 3p14 may be due to the presence of the most common fragile site in the human genome because specific alterations at 3p13-24 have also been detected in a variety of other cancers. At present, no proto-oncogene has been identified at 3p14-21. Therefore, the 3p region may contain one or more "suppressor" or "regulatory" genes which could be either lost or inactivated.

Guinea pig fetal fibroblasts can be transformed to the neoplastic state by either transplacental or *in vitro* exposure to chemical carcinogens. Distinct, extended preneoplastic stages occur during transformation. Thus, the guinea pig model provides an excellent opportunity for studying underlying molecular biology of the carcinogenesis process. Five independently isolated tumorigenic lines initiated by alkylating agents or aryl aromatic hydrocarbons contain activated *ras* oncogenes that transformed NIH/3T3 cells. Activated *ras* genes were not found in preneoplastic progenitor cells. The oncogene of one cell line, 107C3, initiated by MNNG was cloned from both transformed guinea pig cells and from NIH/3T3 transfectants. Clone 107C3/B30 isolated from 3T3/107C3 was capable of transforming NIH/3T3 cells (10 foci/ μ g), whereas clone 107C3/B5 from 107C3 was not. Clone GPB4/B6, homologous to 107C3/B5, was isolated from normal guinea pig cells (GPB4). Ninety-five percent of the bases and 188 of 189 amino acids in the normal human N-*ras* gene matched those in clone 107C3/B5. Thus, the cloned guinea pig sequences contained N-*ras* genes. All three clones contained the codon for asparagine at position 174, while the human N-*ras* gene codes for serine. Therefore, this amino acid difference is due to an evolutionary change. Clone 107C3/B30 differed from the other two clones by a single base in the coding sequences; an AT to TA transversion occurred at the third position of codon 61 resulting in histidine instead of glutamine. This single base mutation accounts for the difference in transforming activity of the clones. Furthermore, the N-*ras* gene in 107C3 cells is heterozygous; one allele contains the CAA codon and the other, the CAT codon. The other four guinea pig cell lines also contained N-*ras* sequences with the identical AT to TA transversion as in 107C3 cells. These results demonstrate that the N-*ras* mutation was not induced by the mutagenic activity of the initiating carcinogen because the same mutation was acquired at a late state of carcinogenesis in five different tumorigenic cell lines, independent of the class of carcinogen used.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP04673-15 LB
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) The Immunobiology of Carcinogenesis		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) P.I.: C. H. Evans Chief, Tumor Biology Section LB NCI Others: S. C. Barnett Visiting Fellow LB NCI B. A. Gelleri Visiting Fellow LB NCI		
COOPERATING UNITS (if any) none		
LAB/BRANCH Laboratory of Biology		
SECTION Tumor Biology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: <div style="text-align: center;">3.5</div>	PROFESSIONAL: <div style="text-align: center;">2.5</div>	OTHER: <div style="text-align: center;">1.0</div>
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Lymphokines, interleukins, and other immunological hormones, i.e., the secretory bioregulatory macromolecules of lymphocytes, macrophages, and other leukocytes are being studied to define their effective anticarcinogenic and tumor cell growth inhibitory activities. Leukoregulin, a newly isolated lymphokine, can prevent carcinogenesis and inhibit tumor cell growth. Anticarcinogenic action is direct, irreversible and occurs without cytotoxicity. Inhibition of tumor cell growth is primarily reversible but can become irreversible due to increased susceptibility of preneoplastic and neoplastic cells to cytolytic destruction by natural killer cells resulting from leukoregulin target cell interaction. Leukoregulin at very high concentrations is also directly cytolytic for tumor cells. The direct acting anticarcinogenic activity of leukoregulin is more potent than the tumor cell inhibitory activity; but by also being able to increase target cell sensitivity to the cytoreductive action of naturally cytotoxic lymphocytes, leukoregulin may be an effective homeostatic mechanism for control of carcinogenesis at its later stages of development. Leukoregulin-induced changes in plasma membrane permeability are accompanied by increased calcium flux and the rapid opening and closing of plasma membrane single ion channels which may be important events in the molecular pathway resulting in inhibition of tumor and other abnormal cell proliferation by this immunologic hormone. Leukoregulin induces identical changes in target cell plasma membrane permeability as occur during natural killer lymphocyte cytotoxicity providing strong evidence that it is an intrinsic mediator or element of the natural cytotoxicity reaction and possibly signifying its central role in immunological homeostasis.		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

C. H. Evans	Chief, Tumor Biology Section	LB	NCI
S. C. Barnett	Visiting Fellow	LB	NCI
B. A. Gelleri	Visiting Fellow	LB	NCI

Objectives:

This project provides a means to study and understand the potential of the normal immune system to prevent, suppress, inhibit and even enhance the growth of an incipient tumor cell during carcinogenesis. Natural cytotoxicity of macrophages, lymphocytes and lymphokines, alone or in combination, are being studied at various stages of carcinogenesis to provide insight into the immunobiology of cancer. As the host mechanisms and the target cell structures with which the immune effectors interact are delineated, it will be possible to investigate how natural and induced immunity may be augmented to suppress and even prevent the final aspects of carcinogenesis--the transition from the preneoplastic to the neoplastic state.

The primary objective of this project is to elucidate at the target cell level the relationship between cell surface alterations accompanying the development of carcinogenesis and host mechanisms capable of preventing, otherwise inhibiting, or even enhancing the development of cancer. Specific objectives include (1) identification of somatic cell alterations during carcinogenesis using *in vitro* model systems to allow study of membrane and other phenotypic changes at specific steps or stages in carcinogenesis and (2) investigation of host interactions with specific cell surface alterations during carcinogenesis. Particular emphasis is placed on natural and induced cellular and humoral immunobiological interactions due to the frequent occurrence of neoantigens, re-expression of fetal antigens, and alterations in allo-antigens on tumor cells.

Methods Employed:

Normal and malignant animal and human cells in culture, including chemical and physical carcinogen-treated cells at progressive stages in the transformation process, are studied for somatic cell changes such as altered morphology, morphological transformation, anchorage-independent growth and tumorigenicity in relation to their interaction and response to components of the immune system. Immunobiological techniques including direct and indirect immunofluorescence, flow cytometry, complement fixation, colony inhibition, radionuclide uptake and release, delayed hypersensitivity skin reactions, and tumor transplantation rejection are employed in analyzing cell membrane changes and in assessing host interactions to the changes. A major emphasis is placed upon flow cytometry and cell sorting to identify plasma membrane and intracellular alterations responsible for regulation of cell proliferation and carcinogenesis.

Major Findings:

Leukoregulin is a recently isolated lymphokine produced by T lymphocytes which is able to prevent as well as inhibit development of radiation or chemical carcinogenesis. Induction of the anticarcinogenic state does not affect cell proliferation and like many responses to hormones the anticarcinogenic state is induced rapidly and persists for a short time. When the leukoregulin anticarcinogenic state is present at the time of carcinogen exposure, the further development of initiated or complete carcinogenesis is irreversibly inhibited.

Later in carcinogenesis when cells have acquired preneoplastic or fully neoplastic properties, leukoregulin is able to inhibit the proliferation of the cells and to increase their sensitivity to natural killer lymphocyte cytotoxicity. These actions are associated with a destabilization of the target cell plasma membrane. The membrane changes are readily detectable by flow cytometric analysis of target cell light scatter and changes in membrane permeability, the latter being followed by the uptake or by the loss of intracellular fluorescent molecules such as fluorescein or propidium iodide, respectively. The same changes are observed in target cells during the course of natural killer lymphocyte cytotoxicity suggesting that leukoregulin may be an intrinsic mediator or element of natural lymphoid cell cytotoxicity and occupy a central role in immunological homeostasis.

The molecular events underlying the perturbation in plasma membrane stability and permeability following target cell interaction with leukoregulin have been studied by contrasting the changes in leukoregulin-treated cells with those after exposure of target cells to a variety of membrane active agents affecting ion transport. Compounds including ouabain, amphotericin B, calcium ionophores A23187 and X-537A, phospholipase C and A2, and phytohemagglutinin, which increase intracellular calcium levels, mimic the membrane changes induced by leukoregulin. Calmodulin, calcium channel blockers and both sodium and potassium ionophores and channel blockers exhibit no flow cytometrically detectable membrane destabilizing activity. The calcium modulators are active over a wide range from 10^{-3} to 10^{-10} M and the one paralleling the kinetic activity of leukoregulin most closely is the calcium ionophore A23187. This suggests that leukoregulin may exert its anti-cancer action in part by increasing intracellular calcium levels. Flow cytometric measurement of intracellular ionic calcium using the calcium binding fluorescent probes quin2 and, more recently, indo-1 reveals a transient increase in intracellular calcium one to ten minutes after treatment of human K562 erythroleukemia cells with leukoregulin. Treatment of the cells with calcium ionophore A23187, however, produces an increase in intracellular calcium within one minute of ionophore exposure indicating that although leukoregulin may increase intracellular calcium levels, it does not function solely as an ionophore. The delay in the rise of intracellular calcium is consistent with leukoregulin binding to its cell surface receptor leading to stimulation of the inositol phospholipid signaling system with a resulting increase in intracellular calcium.

Leukoregulin's alteration of calcium flux and membrane permeability without cytolysis demonstrates that neither event, alone or in combination, is necessarily cytolethal. This is consistent with leukoregulin serving as an immunologic hormone whose function is to condition abnormal cells for eventual elimination by the

host. Modulation of cell surface conformation, membrane fluidity, and permeability to the same degree present in natural lymphocyte-mediated cytotoxicity reactions signifies that leukoregulin through transmembrane signaling has the potential to control the specificity of membrane cytotoxicity by decreasing the proliferative rate of the target cell and increasing the vulnerability of its plasma membrane to additional cytoregulatory agents. Current evidence indicates that tumor and other abnormal cells are more than 10,000-fold more sensitive to the proliferation inhibitory action of leukoregulin than are normal cells.

Cells in all phases of the cell cycle are sensitive to the proliferation inhibitory action of leukoregulin. Flow cytometric analysis of human K562 erythroleukemia cells treated with increasing concentrations of leukoregulin to inhibit more than 90% of the cell replication demonstrates that the percentage of cells in each phase of the cell cycle remains constant during 72 hours of leukoregulin treatment. Fluorescence-activated cell sorting of leukoregulin plasma membrane permeability-altered cells also reveals that cells in each phase of the cell cycle are susceptible to the membrane perturbing action of leukoregulin. Flow cytometric analysis of the sorted cells indicates that cells in the "S" phase of the cell cycle may be more sensitive to the acute membrane destabilizing action of leukoregulin. This cell cycle independent pattern of inhibition of cell replication and its rapid reversibility are quite different from the cell cycle phase-specific blocks observed with the typical inhibitors of cell replication affecting protein and/or nucleic acid synthesis.

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ANNUAL REPORT OF
THE LABORATORY OF CELLULAR CARCINOGENESIS AND TUMOR PROMOTION
CHEMICAL AND PHYSICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1985 through September 30, 1986

The Laboratory of Cellular Carcinogenesis and Tumor Promotion plans, develops and implements a comprehensive research program to determine the molecular and biological changes which occur at the cellular and tissue levels during the process of carcinogenesis. Studies are designed to (1) define normal regulatory mechanisms for cellular growth and differentiation; (2) determine the mechanism by which carcinogens alter normal regulation and the biological nature of these alterations; (3) investigate the mechanism by which tumor promoters enhance the expression of carcinogen-induced alterations; (4) identify cellular determinants for enhanced susceptibility or resistance to carcinogens and tumor promoters; (5) elucidate the mechanism by which certain pharmacologic agents inhibit carcinogenesis.

The Laboratory is composed of three sections each of which is charged with a major responsibility for portions of the Laboratory goals. Because of the integrated approach toward an understanding of mechanisms of carcinogenesis, considerable interaction occurs among the sections. Areas of interaction are defined in the individual project reports.

IN VITRO PATHOGENESIS SECTION: The In Vitro Pathogenesis (IVP) Section (1) develops relevant model systems for the study of all phases of the process of carcinogenesis; (2) defines regulatory mechanisms for the normal control of growth and differentiation and alterations in these controls induced by initiators and promoters; (3) produces, isolates and studies initiated cells; (4) studies functional alterations in gene expression produced by initiators and promoters and the mechanism by which these functional changes occur; (5) elucidates factors which determine susceptibility to carcinogenesis.

This section has directed its efforts toward both developing in vitro model systems to study chemical carcinogenesis in epithelial cells and to use these systems to study the mechanisms of tumor initiation and promotion. Mouse epidermis, the classic model for induction of squamous cancer by chemicals, has been adapted for in vitro study. Previous investigations had demonstrated that this model is a close in vitro analogue of the mouse skin carcinogenesis system in vivo. In vitro, epidermal cells proliferate and differentiate, metabolize carcinogens, repair DNA damage, and respond to tumor promoters like epidermis in vivo.

Regulation of Epidermal Growth and Differentiation and Relationship to Early Events in Epidermal Carcinogenesis: The initiation event in skin carcinogenesis is highly correlated to an alteration in the program of terminal differentiation of epidermal cells. In cell culture, epidermal differentiation is regulated by the concentration of extracellular Ca^{2+} . Ca^{2+} concentrations which induce epidermal differentiation activate phospholipase C which catalyzes the metabolism of phosphatidylinositol. The consequent generation of diacylglycerol activates protein kinase C which serves as a second messenger in epidermal differentiation.

Epidermal differentiation is associated with the modulation of expression of specific genes, including a gene coding for a precursor protein for cornified envelope assembly and a protease inhibitor gene. The expression of these genes is also modified in epidermal tumors. The ras oncogene is highly correlated to the initiated phenotype in epidermis. Introduction of an activated ras gene into normal keratinocytes converts them into papilloma cells. Chemically induced papillomas yield an activated ras oncogene with a mutation at codon 61. The activation of a ras oncogene in papilloma cells is associated with the expression of a unique keratin protein which is normally expressed only in simple epithelia. Papilloma cells and initiated cells are resistant to the differentiation-inducing effects of phorbol ester tumor promoters and to the cytotoxic effects of benzoyl peroxide. Since phorbol esters induce differentiation in normal cells, papilloma cells can be selected among an excess of normal cells in culture by their ability to continue to proliferate in culture medium containing phorbol esters. In vivo, several classes of benign tumors can be induced by initiation and promotion. Tumors can be subdivided into those with a high risk for malignant progression and those with a very low risk for malignant progression. Future studies are designed to determine whether a specific set of genes, which predetermine the biologic potential of the tumor cell, is activated during initiation.

Molecular Regulation of Epidermal-Specific Differentiation Products: cDNA clones corresponding to the major keratins expressed in mouse epidermis have been isolated and characterized. Using a combination of in situ hybridization with RNA probes, which are specific for individual keratin mRNAs, and indirect immunofluorescence with monospecific antisera, which were elicited with synthetic peptides corresponding to unique sequences within each keratin subunit, it is possible to show that these keratin genes belong to at least three subsets: those expressed predominantly in the proliferating basal layer of the epidermis, those expressed predominantly in the differentiated suprabasal layers of the epidermis, and those only expressed under hyperproliferative conditions such as in benign and malignant epidermal tumors, hyperplasia induced by the tumor promoter TPA and squamous metaplasia induced by various means in hamster trachea. Genes representing each subset have been isolated and sequenced. Various strategies have been employed to identify sequences regulating the expression of these genes including vector construct using different regions of the genomic clones to drive expression of the neomycin-resistance gene and the chloramphenicol acetyl transferase gene and the production of transgenic mice containing genomic fragments encoding human keratins. The in situ hybridization technique has been used to confirm previous results obtained with monospecific antisera which demonstrates that malignant epidermal tumors could be distinguished from benign tumors due to their failure to express the differentiation-associated keratins. The in situ hybridization technique has also been used to study the expression of human keratin genes in epidermal tumors and other skin disorders. In addition, this technique has been used to localize transcripts of other genes that are differentially expressed in the epidermis.

Determinants for Susceptibility to Carcinogenesis: In vivo studies have shown that the SENCAR mouse is unusually sensitive to skin carcinogenesis by initiation and promotion while the Balb/c mouse is resistant. Techniques were developed for grafting epidermal and dermal cells to athymic nude mice in order to form a reconstituted skin. Cell lines were established in culture from papillomas produced on both SENCAR (SP lines) and Balb/c (BP lines) mice in

vivo by initiation with DMBA and promotion with 12-O-tetradecanoylphorbol-13-acetate (TPA). When grafted to nude mice, these lines produce papillomas. Cell line 308RB is a differentiation-resistant cell line established from initiated Balb/c mouse skin by selection in high Ca^{2+} medium and this line also produces papillomas in vivo by grafting. Line SP-1, the most extensively studied to date, produces papillomas whose size increases as the number of cells grafted increases from 16,000 to 1,600,000. Addition of normal primary epidermal cells at the time of grafting suppresses papilloma formation by SP-1 cells in proportion to the number of normal cells added. The development of these cell lines provides a means for controlled study of the suppression of Balb/c and SENCAR papilloma development by homologous and heterologous normal primary epidermal cells and the overcoming of this suppression in response to tumor promotion. Furthermore, these cell lines are a useful model for study of the conversion of benign to malignant tumor cells.

Immunological Techniques to Study the Interaction of Carcinogens with DNA:

Antibodies specific for carcinogen-DNA adducts have probed the nature, extent, and consequences of in vitro and in vivo DNA modification. DNAs substituted with 2-acetylaminofluorene (AAF) and cis-diaminedichloroplatinum II (cis-DDP) were analyzed by quantitative immunoassays able to detect about 100 adducts per cell and by immunohistochemical procedures developed to localize adducts in situ. In hepatic DNA of rats fed a carcinogenic dose of AAF for 4 weeks, adduct accumulation reached a plateau at 2-3 weeks. Since adduct removal was biphasic during 4 subsequent weeks on control diet, a computer-derived pharmacokinetic model proposed that adducts are formed into two genomic compartments, one from which they are removed rapidly and another from which they are removed slowly. Studies initiated to identify these two compartments involve adduct analysis in different liver cell types and different chromatin regions including the nuclear matrix. Immunohistochemical localization of AF-DNA adducts in livers of rats fed AAF was shown by fluorescence and microfluorometry to be concentrated primarily in periportal regions. In addition, there were no adducts detectable by immunofluorescence in preneoplastic foci induced by several different protocols. Nucleated peripheral blood cell DNA was obtained from cancer patients at multiple times during courses of cis-DDP therapy, and a total of 254 samples were analyzed. Of these, 23 untreated control samples were negative, and 43% of the 231 samples from patients receiving cis-DDP were positive. Adduct accumulation, in positive patients, occurred as a function of total cumulative dose, suggesting relatively slow adduct removal. Disease response data on 55 patients indicated that individuals with high adduct levels have a high rate of complete response to therapy. Parallel experiments in animal models have demonstrated that the same adduct forms in kidney, gonads, and tumors of rats and mice in direct relation to dose.

DIFFERENTIATION CONTROL SECTION: The Differentiation Control Section (1) studies the biological and biochemical factors involved in normal differentiation of epithelial tissues; (2) uses pharmacological techniques to alter differentiation of normal, preneoplastic and neoplastic epithelial cells to determine the relevance of differentiation to carcinogenesis and to determine methods to intervene in preneoplastic progression; (3) studies the relationship between differentiation and growth control; (4) focuses on cell surface changes in differentiation and neoplasia.

Vitamin A and its derivatives, the retinoids, are of interest in cancer research because they play an essential role in the maintenance of normal differentiation in most epithelial tissues, under normal physiological conditions. At the biochemical level our laboratory has demonstrated that retinol and retinoic acid regulate membrane glycosylation reactions. Such biochemical involvement is consistent with the reported alterations in glycosylation of $\alpha_2\mu$ -globulin and α_1 -macroglobulin in vitamin A-deficient livers, and it might explain altered oligosaccharide composition of fibronectin secreted by chick sternal chondrocytes cultured in excess retinoic acid.

Vitamin A as a Regulator of the Dolichylphosphate-mediated Pathway of Protein Glycosylation: Work from our laboratory has demonstrated that the condition of vitamin A deficiency causes a marked (up to 95%) decrease in the incorporation of 2-[^3H]mannose into glycoproteins in vivo. The entire pathway from mannose to mannose-phosphate, guanosine diphosphomannose, dolichylphosphate mannose, lipid-linked oligosaccharides and glycoproteins was investigated to determine the steps controlled by the vitamin A status in hamster liver. The primary effect was shown to be on the biosynthesis of GDP-mannose. Since enzyme activity responsible for GDP-mannose synthesis from GTP and mannose phosphate was not influenced by vitamin A deficiency and mannose phosphate was found to accumulate, it appears that lower concentrations of GTP are responsible for the observed effect of vitamin A deficiency. These findings were confirmed in organ culture of hamster trachea, where deficiency caused an accumulation of mannose phosphate and a decrease in GDP-mannose labeling, using 2-[^3H]mannose as the precursor.

Studies on Retinoid Transport: Serum retinol binding protein (RBP) is responsible for the transport of retinol from the liver to other vitamin A requiring target cells such as the epidermis. Several studies suggest that RBP is recognized by target cells through a specific cell surface receptor. We have used primary mouse epidermal cells as a model system to more clearly delineate the steps involved in the delivery of retinol from RBP to target cells. We have purified RBP from rat serum, loaded it with [^3H]retinol, and purified the [^3H]retinol-RBP complex by affinity chromatography on human prealbumin Sepharose. Epidermal cells incubated with [^3H]retinol-RBP complex accumulate cell-associated radioactivity in a time-dependent manner. The uptake of [^3H]retinol from the [^3H]retinol-RBP complex was inhibited by unlabeled holo-RBP with an apparent K_m of 2-4 μM , the concentration at which RBP is normally found in serum. [^3H]Retinol uptake from RBP was not influenced by inhibitors of receptor-mediated endocytosis and, using ^{125}I -labeled RBP, no evidence of direct binding or internalization of RBP was found. Homogenization and centrifugation of cells following delivery of [^3H]retinol from RBP determined that 80% of the cell-associated radioactivity was membrane bound. These results are consistent with retinol being delivered from RBP to epidermal cells via a transitory interaction with a cell surface receptor.

Retinoid Status and the Control of Differentiation: Formation of Dental Masses in the Upper Incisors of Rats Kept on Dietary Cycles of Vitamin A-Deficient and Retinoic Acid-Supplemented Diets: Cyclic dietary regimens of 18 days on a retinoic acid-containing diet followed by 10 days on a vitamin A-deficient diet have been used in rats to provide animals which are synchronized as far as A-deficiency status and relatively free of bacterial infection. We found that maintenance of male Sprague-Dawley rats on this cyclic dietary regimen for more than 330 days caused the formation of "dental masses" in the upper incisor teeth

in the majority of the rats (60-70%). The lesions were composed of cords of odontogenic epithelium surrounded by a mantle of mesenchyme. In nearly all cases the predentin was accumulated around a focus of keratinized epithelium. These tumor-like masses do not appear to be neoplastic, but represent ectopic transplants of odontogenic epithelium and associated mesenchyme that respond functionally to vitamin A.

MOLECULAR MECHANISMS OF TUMOR PROMOTION SECTION: The early events in the interaction of the phorbol esters with cells and tissues are being characterized. Specific aims are as follows: 1) Analysis of the functional domains of protein kinase C in vitro; 2) determination of the biological activities of intact protein kinase C and its functional domains upon microinjection back into cells; 3) elucidation of mechanistic differences between different classes of protein kinase C activators; 4) understanding of the role of diglycerides and protein kinase C in keratinocyte differentiation and tumor promotion; 5) identification of targets for the phorbol esters and related diterpenes in addition to protein kinase C. The major phorbol ester receptor, protein kinase C, is postulated to mediate one of the two pathways activated by a large class of hormones for which receptor occupancy is associated with rapid phosphatidylinositol turnover. Several oncogenes may also function, in part, through this pathway. Understanding of the mechanisms of endogenous modulation of the phorbol ester receptors may thus provide insights into both basic biochemical mechanisms and the process of human carcinogenesis as well as to identify biochemical steps suitable for intervention.

Most of the evidence for the role of protein kinase C in phorbol ester action is indirect. Its involvement in the mitogenic response of Swiss 3T3 cells was demonstrated directly by microinjection, using Swiss 3T3 cells down-regulated by chronic phorbol ester treatment. Emerging evidence suggests prominent proteolytic processing of protein kinase C. The catalytically active fragment of the enzyme was prepared by tryptic digestion and was characterized. Phospholipids regulated its activity in a pH- and substrate-dependent fashion, distinct from the activation of the intact enzyme by the interaction of phospholipids at the regulatory domain. The regulatory domain, being devoid of enzymatic activity and thus a pseudo-receptor, was also generated and is now being characterized. Analysis of modulators of protein kinase C other than the phorbol esters afford unique insights into its function. Bryostatins, macrocyclic lactones, activate protein kinase C and compete for phorbol ester binding. Paradoxically, they blocked phorbol ester action in two differentiating systems (HL-60 promyelocytic leukemia cells and Friend erythroleukemia cells) and blocked some, but not other, responses in keratinocytes. In HL-60 cells, the bryostatins induced a similar, but distinct, pattern of phosphorylation. Diglycerides are the postulated endogenous analogs of the phorbol esters. In support of this hypothesis, treatment of mouse keratinocytes with bromooctanoic acid, which blocks triglyceride formation and causes diglyceride accumulation, induced effects similar to the phorbol esters. Bromooctanoic acid may provide a tool for assessing the tumor promoting activity of the diglycerides. Modeling studies comparing different classes of protein kinase C activators indicated putative common features. Novel structures incorporating these features inhibited phorbol ester binding and induced typical phorbol ester responses such as inhibition of EGF binding or phosphorylation of a 40 kd platelet protein.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04504-14 CCTP

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Model Systems for the Study of Chemical Carcinogenesis at the Cellular Level

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S. H. Yuspa Chief LCCTP NCI

Others: H. Hennings Research Chemist LCCTP NCI
M. Poirier Research Chemist LCCTP NCI
D. Roop Senior Staff Fellow LCCTP NCI
J. Strickland Research Chemist LCCTP NCI
U. Lichti Expert LCCTP NCI
J. Harper Staff Fellow LCCTP NCI

COOPERATING UNITS (if any)

Microbiological Associates, Bethesda, MD (E. F. Spangler)

LAB/BRANCH

Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION

In Vitro Pathogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

9.0

PROFESSIONAL:

5.0

OTHER:

4.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Cellular and molecular aspects of chemical carcinogenesis in lining epithelia are studied in mouse epidermis by in vivo and in vitro techniques. The initiation event in skin carcinogenesis is highly correlated to an alteration in the program of terminal differentiation of epidermal cells. In cell culture, epidermal differentiation is regulated by the concentration of extracellular Ca^{2+} . Ca^{2+} concentrations which induce epidermal differentiation activate phospholipase C which catalyzes the metabolism of phosphatidylinositol. The consequent generation of diacylglycerol activates protein kinase C which serves as a second messenger in epidermal differentiation. Epidermal differentiation is associated with the modulation of expression of specific genes, including a gene coding for a precursor protein for cornified envelope assembly and a protease inhibitor gene. The expression of these genes is also modified in epidermal tumors. The ras oncogene is highly correlated to the initiated phenotype in epidermis. Introduction of an activated ras gene into normal keratinocytes converts them into papilloma cells. Chemically induced papillomas yield an activated ras oncogene with a mutation at codon 61. The activation of a ras oncogene in papilloma cells is associated with the expression of a unique keratin protein which is normally expressed only in simple epithelia. Papilloma cells and initiated cells are resistant to the differentiation-inducing effects of phorbol ester tumor promoters and to the cytotoxic effects of benzoyl peroxide. Since phorbol esters induce differentiation in normal cells, papilloma cells can be selected among an excess of normal cells in culture by their ability to continue to proliferate in culture medium containing phorbol esters. In vivo, several classes of benign tumors can be induced by initiation and promotion. Tumors can be subdivided into those with a high risk for malignant progression and those with a very low risk for malignant progression. Future studies are designed to determine whether a specific set of genes, which pre-determine the biologic potential of the tumor cell, is activated during initiation.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. Yuspa	Chief	LCCTP	NCI
H. Hennings	Research Chemist	LCCTP	NCI
M. Poirier	Research Chemist	LCCTP	NCI
D. Roop	Senior Staff Fellow	LCCTP	NCI
J. Strickland	Research Chemist	LCCTP	NCI
U. Lichti	Expert	LCCTP	NCI
J. Harper	Staff Fellow	LCCTP	NCI
S. Jaken	Senior Scientist	FDA	NIH
D. Lowy	Chief	LCO	NCI
S. Nagae	Visiting Fellow	LCCTP	NCI
P. Steinert	Visiting Scientist	DB	NCI
S. Aaronson	Chief	LCMB	NCI

Objectives:

To study cellular and molecular changes during stages of chemical carcinogenesis through the development and use of cultures of epithelial lining cells which are the major target site for cancer in humans. Studies are directed to give insight into general changes occurring in specialized mammalian cells during malignant transformation and specific molecular events which may be causative to the transformation process. Specific markers of the transformed phenotype of epithelia are also being sought and mechanisms to prevent or reverse transformation are being studied.

Methods Employed:

This laboratory has developed and utilized mouse epidermal cell culture as an appropriate model to approach the stated objectives. Previous studies have shown that this model functions biologically in a fashion highly analogous to mouse skin in vivo. Human epidermal cells obtained from neonatal foreskins have also been adapted to growth in vitro. In vivo studies utilizing the initiation-promotion model for mouse skin carcinogenesis and grafts of human or mouse skin or cultured cells onto nude mice are also employed. A number of laboratory techniques are required to pursue the objectives. Morphology is followed by light and electron microscopy and immunohistochemical staining. Macromolecular synthesis and growth kinetics are studied by biochemical and autoradiographic procedures and flow cytometry. Intracellular ion changes are assayed by atomic absorption spectrometry. Cellular metabolic functions, including the production of specific differentiation products, are monitored by enzyme assays, one- and two-dimensional gel electrophoresis, amino acid analysis, and radioimmunoassay. Protein purification techniques employ column chromatography, fast protein liquid chromatography, and high pressure liquid chromatography. The progression to the malignant phenotype is monitored by growth rates, soft agar assay, karyotypic abnormalities, enzymatic changes, changes in gene expression at the level of mRNA and injection of cells into nude or newborn mice. Genetic aberrations are studied by DNA transfection, gene cloning, nucleic acid hybridization and restriction analysis.

Major Findings:

This project has provided new information on the regulation of the normal program of differentiation in epidermis, the mechanism of action of pharmacological modifiers of normal differentiation, and aberrant differentiation during the process of carcinogenesis. In epidermal cell culture, extracellular Ca^{2+} concentrations greater than 0.1 mM induce terminal differentiation. This can be inhibited by lanthanum, suggesting that Ca^{2+} influx or Ca^{2+} at the cell surface is responsible for the differentiation signal. A large increase in the metabolism of phosphatidylinositol occurs within seconds of Ca^{2+} exposure resulting in an increase of inositol phosphates and diacylglycerol intracellularly. Phosphatidylinositol turnover is also increased by A23187, a calcium ionophore, suggesting that Ca^{2+} influx stimulates metabolism of this phospholipid. The induction of epidermal differentiation by Ca^{2+} results in the cross-linking of specific intracellular proteins to form a cornified envelope. Amino acid analysis of mature cornified envelopes indicates they are glycine- and serine-rich. Immature cornified envelopes have considerably less glycine and serine content, suggesting that a glycine/serine-rich protein is added to cornified envelopes during later stages of differentiation. A gene coding for a glycine/serine-rich protein has been isolated from an epidermal cDNA expression library and sequenced. Antibodies generated against three synthetic peptides, corresponding to C-terminal regions of the deduced amino acid sequence, immunoprecipitate a protein of about 50 kd. The same protein appears to be immunoprecipitated by antibodies to purified cornified envelope. Thus the glycine/serine-rich protein appears to be an important component of the differentiation program and a useful marker for the later stages of differentiation. The anti-cornified envelope antibody also produces diffuse staining of high molecular weight proteins, probably partially cross-linked, in Western blots of particulate proteins from maturing cells. Particulate proteins from epidermal cells exposed to retinoic acid, which blocks the cornification process, do not contain proteins recognized by the cornified envelope antibody. Proteases appear to play a regulatory role in epidermal differentiation and may be important in the activation of epidermal transglutaminase. A specific protease inhibitor gene has been isolated from an epidermal cDNA expression library and sequencing of the cloned gene has revealed the amino acid sequence of the protein. Epidermal cells express a single 1.9 kb transcript of this gene which is abundant in basal cells but diminishes during terminal differentiation. In situ hybridization studies with the cloned cDNA probe indicates high transcriptional activity in basal and spinous cells which diminishes in granular cells where the cornified envelope is assembled. The protease inhibitor protein appears to persist in the more differentiated cells where its active synthesis is diminished. The potential role for this protease inhibitor in epidermal differentiation is currently being explored.

Considerable evidence has accumulated which indicates that a number of carcinogen derived tumors of skin arise from hair follicle cells. A model system for the three-dimensional growth of hair follicles in collagen gels has been developed. Hair follicles can be stimulated to proliferate by exposure to cholera toxin and to secrete collagenase by exposure to epidermal growth factor. When removed from culture and grafted to nude mice, hair follicles produce a completely intact haired skin. Experiments are currently underway to try to induce neoplastic change in cultured hair follicles.

The epidermal cell culture model has revealed that a defect in the response to the Ca^{2+} signal for differentiation is an early event in chemical carcinogenesis. A number of Ca^{2+} -resistant cell lines have been derived and many of these have been tested recently by grafting viable cells to a prepared graft site on nude mice (see Project Z01CP05178-05 CCTP). One tested line has yielded papillomas upon grafting, while a number of others have produced carcinomas. Cell line 211 formed an adenosquamous carcinoma upon grafting, suggesting that this cell line was of hair follicle origin. When a mutated Harvey ras oncogene is introduced into normal keratinocytes by a defective retroviral vector, the resultant cells are resistant to Ca^{2+} -induced differentiation and produce papillomas when grafted back to nude mice. In situ hybridization studies with radiolabeled probes to the activated ras oncogene reveal that the exogenous ras gene is expressed only in the most basal cells of the benign tumors and transcripts are not apparent beyond the first suprabasal cell layer. The ability to regulate expression of the exogenous oncogene could play a role in the benign behavior of the tumor. The majority of papillomas induced in vivo by DMBA initiation and TPA promotion contain an activated ras oncogene with a mutation in codon 61. Several papilloma cell lines derived from chemically induced tumors also contain an activated ras oncogene although we have also derived cell lines from papillomas which do not carry a transforming oncogene for NIH 3T3 cells. In cell culture, epidermal cells containing an activated ras gene express a 58 kd keratin which is not normally expressed in the epidermis or other stratified squamous epithelia. This keratin is characteristic of simple epithelia but may be a marker for squamous tumors resulting from the activation of a ras oncogene.

Tumor promotion in mouse skin appears to be a process of cell selection in which initiated cells are provided a growth advantage. Phorbol ester tumor promoters induce epidermal differentiation, but initiated cells or papilloma cells are resistant to this influence of tumor promoters. When normal epidermal cells were subdivided into basal and suprabasal cell types, the suprabasal cell types were particularly sensitive to the induction of differentiation by phorbol esters. The majority of phorbol ester-resistant initiated and papilloma cell lines show cross-resistance to the cytotoxic effects of benzoyl peroxide, suggesting a mechanism of promotion for that agent which is highly cytotoxic to normal epidermal cells. The differential sensitivity of papilloma cells and normal cells for the induction of terminal differentiation by phorbol esters was used as an in vitro model for tumor promotion. A small number of papilloma cells was mixed with an excess of normal cells to recreate the cell populations of initiated skin. When such mixed cultures are treated with phorbol ester tumor promoters, the papilloma cells selectively expand into large foci. In the absence of phorbol ester exposure, the growth of the papilloma cells is suppressed by the excess of normal cells.

In vivo experiments on mouse skin, using the second stage promoter mezerein on initiated mice, have indicated that the first stage of tumor promotion occurs spontaneously by 10 weeks after initiation. Tumors promoted by mezerein starting after a 10 week interval from initiation have a high risk for converting to carcinomas, suggesting that such tumors are derived from initiated cells of a specific subclass. The promotion of papillomas with a high risk for malignancy was also observed with deoxyphorbolisobutyrate, a phorbol derivative which

is a weak promoter and produces only a few benign tumors. Future studies are designed to distinguish papillomas at high risk for malignancy from tumors at low risk in order to determine whether a specific set of genes is activated during initiation which predetermine the biological potential of the tumor cell.

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Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP04798-16 CCTP
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Metabolism and Mode of Action of Vitamin A		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	L. M. DeLuca	Research Chemist LCCTP NCI
Others:	K. E. Creek D. Rimoldi S. Kato E. M. McDowell	Staff Fellow Visiting Fellow Visiting Fellow IPA Appointee LCCTP NCI LCCTP NCI LCCTP NCI LCCTP NCI
COOPERATING UNITS (if any) Microbiological Associates, Bethesda, MD (R. Shores and E. F. Spangler)		
LAB/BRANCH Laboratory of Cellular Carcinogenesis and Tumor Promotion		
SECTION Differentiation Control Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 7.0	PROFESSIONAL: 5.0	OTHER: 2.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Maintenance of epithelial differentiation is one of the biological functions of vitamin A and explains current interest in this nutrient as a chemopreventative agent of epithelial cancer. Therefore, current research efforts center on the elucidation of possible sites and mechanisms of action of the vitamin. These were pursued at the following levels: 1) Protein glycosylations: The key intermediates in the pathway of mannose incorporation into glycoproteins were studied to identify vitamin A dependent steps. An accumulation of mannose phosphate and a concomitant decrease in GDP-mannose formation were found in liver <u>in vivo</u> and cultured tracheas from vitamin A-deficient hamsters. Since the activity of the enzyme responsible for GDP-mannose synthesis from GTP and mannose phosphate was not affected, lowered GTP pools may be responsible for the effect of vitamin A deficiency on mannose incorporation. 2) Retinol transport: The possibility that transport of retinol to mouse epidermal cells is mediated via a cell surface receptor for the plasma retinol binding protein was tested, utilizing [³ H]retinol-labeled RBP and cultured mouse epidermal cells. The uptake of [³ H]retinol was inhibited by holo-RBP with an apparent Km of 2-4 μM, the concentration at which RBP is found in the serum. [³ H]Retinol uptake from RBP was not influenced by inhibitors of receptor mediated endocytosis and using ¹²⁵ I-labeled RBP no evidence of direct binding or internalization of RBP was found. These data are consistent with retinol being delivered from RBP to epidermal cells via a transitory interaction with a cell surface receptor. 3) Effect of dietary cycles of vitamin A deficiency and retinoic acid depletions on incisor tooth of the rat. The majority of rats (60-70%) maintained for more than 11 cycles on alternating dietary regimens of vitamin A-deficient (10 days) and retinoic acid-supplemented (18 days) diets developed incisor "tooth masses." These masses appeared to originate from ectopic epithelial and mesenchymal cells which had crossed the dentin wall and responded to intermittent availability of retinoic acid in the diet.		

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

L. M. De Luca	Research Chemist	LCCTP NCI
K. E. Creek	Staff Fellow	LCCTP NCI
D. Rimoldi	Visiting Fellow	LCCTP NCI
S. Kato	Visiting Fellow	LCCTP NCI
E. M. McDowell	IPA Appointee	LCCTP NCI

Objectives:

Vitamin A and some of its synthetic analogs (retinoids) have been shown to act as chemopreventative agents during the promotion phase in experimentally-induced epithelial cancer in several animal model systems. Therefore, an investigation of their biochemical mode of action may reveal key points in our understanding of the biochemical steps involved in the control of tumor promotion. Such investigation was conducted along the following lines:

- A. To determine the mechanism by which retinoids influence the glycosylation of proteins in hamster liver in vivo and in cultured hamster trachea.
- B. To study retinoid transport and metabolism in cultured mouse epidermal cells.
- C. To define lesions arising in rats maintained on a cyclic retinoic acid-supplemented and- depleted diet.

Methods Employed:

Vitamin A deficiency was induced in Syrian golden hamsters by placing the mothers on a mixed diet (Purina lab chow/vitamin A-free diet, 1:1) 2 to 5 days prior to birth of the experimental animals, after which only the vitamin A-free diet was used for both nursing mothers and experimental animals, which were weaned on day 21. Retinoic acid-repleted hamsters received the same vitamin A-free diet supplemented with all-trans retinoic acid (30 µg/g).

A similar protocol was used to obtain vitamin A-deficient male Sprague-Dawley rats. These rats were then switched to a diet containing 2 µg of retinoic acid/g and kept for 18 days on this diet. They were then switched back to a vitamin A-deficient diet. This cycle was repeated up to 14 times.

HPLC Analysis of Methanolic Extracts of [2-3H]Mannose-Labeled Hamster Liver Homogenate.

HPLC analyses were performed on an Altex Model 100A programmable liquid chromatography system, the radioactivity was monitored by a Flo-One Model HS radioactivity flow detector, and the data recorded by a Linear chart recorder. Methanolic extracts from liver homogenates of hamsters injected with 2-[³H]-mannose were chromatographed on an anion exchange Mono Q HR 5/5 column using a

system which separates mannose, mannose phosphate, and GDP-mannose. Samples containing about 150,000 cpm in up to 1.5 ml of 70% methanol were loaded onto the Mono Q column which was eluted with a linear gradient of NaCl in 70% methanol. Typical retention times for standard mannose, mannose phosphate and GDP-mannose were 2.6, 13.7, and 19.0 min, respectively.

Dol-P-Man, Lipid-Linked Oligosaccharide and Glycopeptide Analysis of [2-3H]-Mannose-Labeled Hamster Liver Post-Nuclear Membranes.

The post-nuclear membrane suspension (1.0 ml) was extracted with two volumes of chloroform/methanol (2:1, v/v). The aqueous and organic phases were separated by centrifugation for 5 min in a clinical type centrifuge, the lower organic phase was removed, dried under a stream of nitrogen and dissolved in 1.5 ml of chloroform/methanol (2/1, v/v). This extract (approximately 20,000 cpm) was diluted to 14 ml with 99% methanol and applied to a 1 ml Econo-Column of DEAE-Sephacel equilibrated in 99% methanol. After washing with 9 ml of 99% methanol, Dol-P-Man was eluted with 7 ml of 30 mM ammonium acetate in 99% methanol. Fractions of 1 ml were collected and aliquots monitored for radioactivity. Total recovery of radioactivity from the column was 85-100%.

The denaturated protein pellet which partitions at the interface of the aqueous and organic phases after chloroform/methanol (2:1, v/v) extraction was washed twice with chloroform/methanol (2:1, v/v) (4.0 ml), three times with water (3.0 ml) and the sample then lyophilized. Lipid-linked oligosaccharides were extracted from the lyophilized pellet with chloroform/methanol/water (1:1:0.3, v/v) three times (3.0 ml). An aliquot of the chloroform/methanol/water (1:1:0.3, v/v) extract was monitored for radioactivity, which represents the amount of [2-3H]mannose label incorporated into lipid-linked oligosaccharides. The chloroform/methanol/water extract was hydrolyzed with mild acid and analyzed on a Spherisorb-NH₂ column eluted with a gradient of acetonitrile/H₂O. This procedure allows the separation of oligosaccharides based on size.

The residue remaining after the chloroform/methanol/water (1:1:0.3, v/v) extraction was digested with Pronase to generate glycopeptides.

Assay of GDP-Mannose Pyrophosphorylase.

GDP-mannose pyrophosphorylase was assayed in liver cytosol. A mixture (100 μ l) containing 50 mM Hepes buffer, 2.5 mM MgCl₂, pH 7.5, 0.2 mg/ml BSA, 3 mM AMP, 8 mM NaF, 67 milliunits of inorganic pyrophosphatase and 500 μ M GTP was added to a microfuge tube containing 5×10^4 cpm of D-[¹⁴C]mannose 1-phosphate (final concentration about 0.92 μ M). After mixing, the reaction was started by addition of 4-6 μ l (2.0-2.5 μ g of protein) of 200 times-diluted 105,000 x g supernatant (see preparation of post-nuclear membranes). After 15 min of incubation at 37°C the reaction was stopped by adding methanol (250 μ l) and the amount of GDP-mannose formed was quantitated by HPLC analysis of the methanolic solution on a Mono Q column with the system described above.

Other Methods:

Procedures to culture hamster tracheas and mouse epidermal cells have been published.

Major Findings:A. Background and Rationale of Glycosylation Studies

Vitamin A and its derivatives (collectively termed retinoids) play an essential role in the maintenance of normal cellular differentiation and proliferation. Numerous studies in several model animal systems have shown that retinoids can act as chemopreventive agents during the promotion phase of experimentally induced epithelial cancer. Furthermore, retinoids are important for growth of normal cells and have been shown to modify growth and adhesive properties of transformed cultured cells in the direction of the normal phenotype.

Studies conducted in a variety of tissues indicate that in normal physiology the vitamin may, somehow, be involved in the glycosylation of mannose-containing glycoconjugates. Livers from vitamin A-deficient animals show a decrease in mannose incorporation into glycoproteins, while excess vitamin A enhanced incorporation of mannose, but not galactose, into liver glycoconjugates. We found that nutritional deprivation of vitamin A produced an early, reproducible and profound decrease in the incorporation of [2-³H]mannose into GDP-mannose. As a consequence of this decrease, the entire pathway of dolichylphosphate-mediated mannosylation was affected. On the contrary mannose phosphate accumulated as deficiency became more severe.

Further, we analyzed the synthetic enzyme activity (GDP-mannose pyrophosphorylase) responsible for GDP-mannose synthesis from GTP and mannose phosphate and found it unchanged by vitamin A deficiency, thus indicating that lower GTP levels are probably responsible for the drop in GDP-mannose synthesis during vitamin A deficiency.

Hamster tracheas from animals maintained for 4 weeks on a vitamin A-deficient diet or the same diet supplemented with 30 µg of RA/g and cultured for a fifth week in the presence or absence of 10⁻⁸ M RA clearly showed a profound decrease in [2-³H]mannose incorporation into GDP-mannose, while mannose phosphate was enhanced in the vitamin A-deficient tracheas.

The conclusion of this work would suggest lowered GTP levels as the main responsible parameter in the decreased glycoprotein mannosylation in vitamin A deficiency.

B. Retinoid Transport.

Little information exists as to the mechanism by which retinol and/or retinoic acid is delivered from serum retinol binding protein (RBP) and enters in vitamin A-responsive cells. Furthermore, once it enters the cell, the intracellular pathway the vitamin follows or even the exact subcellular compartment(s) in which it exerts its action are unknown. Primary mouse epidermal cells exhibit an induction of transglutaminase activity and an inhibition of cornified envelope formation following exposure to retinoic acid. Since the responsiveness of epidermal cells to retinoic acid is well documented, this cell system was used to identify specific receptor molecules at the cell surface for the retinol binding protein (RBP).

Epidermal cells incubated with free [^3H]retinol or [^3H]retinol-RBP complex accumulated radioactivity in a time-dependent manner. Cells incubated with free [^3H]retinol acquired 15-fold the amount of radioactive ligand than if the retinol was delivered via RBP. Addition of a several-fold molar excess of unlabeled retinol did not inhibit the uptake of free [^3H]retinol or [^3H]retinol bound to RBP. In contrast, the uptake of [^3H]retinol from the [^3H]retinol-RBP complex was inhibited by unlabeled holo-RBP with an apparent K_m of 2-4 μM , the concentration at which RBP is normally found in serum.

Inhibitors of receptor-mediated endocytosis did not influence [^3H]retinol uptake from RBP. Moreover, using ^{125}I -labeled RBP, neither direct binding or internalization of RBP was observed. Of the cell associated [^3H]retinol, 80% was found in the membrane. Therefore, RBP may deliver retinol to epidermal cells via a transitory interaction with a cell surface receptor. Another functional aspect of RBP may be to protect cells from a nonspecific and rapid uptake of the vitamin by biological membranes, which occurs when retinol is not bound to RBP.

C. Retinoid Status and the Control of Differentiation: Formation of Dental Masses in Upper Incisors of Rats Kept on Dietary Cycles of Vitamin A-Deficient and Retinoic Acid-Supplemented Diets.

The hypothesis that vitamin A deficiency may be a contributory factor to tumor development is difficult to test because the experimental animals usually die from infection in less than 10 weeks after they are placed on a vitamin A-deficient diet. However, it is well known that cycles of RA supplementation (18 days) and depletion (10 days) permit the induction of rapid, synchronous and repeated vitamin A deficiencies in marginally vitamin A-sufficient rats, according to published procedures. This work was conducted in an effort to reproduce the effect in male Sprague-Dawley rats, and if demonstrated, to eventually apply it to experimentation where the creation of cycles of vitamin A deficiency could be tested as a potential tumor promoter in various organs.

Maintenance of the rats on this cyclic dietary regimen for more than 330 days caused the formation of "dental masses" in the upper incisor teeth in 61 out of 100 rats. The lesions were composed of cords of odontogenic epithelium surrounded by mesenchyme. In all lesions the epithelium was simple cuboidal, consisting of branching cords of poorly differentiated cells resting on a basement membrane. Some cells were in mitosis. The epithelial cords were keratinized focally and small keratin pearls were distributed randomly. However, these tumor-like masses do not appear to be neoplastic. Instead they appear to derive from the growth of ectopic odontogenic epithelium and associated mesenchyme that respond functionally to retinoic acid. With repeated dietary cycles it is expected that the expanding pulpal mass would herniate through perforations in the dentin wall and pass into the surrounding connective tissue. The dental masses arose because a normally continuously growing tissue (pulpal mesenchyme and the associated odontogenic epithelium) continued to grow during periods of vitamin A repletion in an ectopic site where functional attrition from mastication was not occurring.

Publications:

Clifford, A. J., Tondeur, Y., Creek, K. E., Silverman-Jones, C. S. and De Luca, L. M.: FAB and collisional activation mass spectrometry of retinyl phosphate mannose synthesized by liver membranes. Biomed. Mass Spectrom. 12: 221-228, 1985.

Creek, K. E., Rimoldi, D., Clifford, A. J., Silverman-Jones, C. S. and De Luca, L. M.: Mannosylation of endogenous and exogenous phosphatidic acid by liver microsomal membranes: formation of phosphatidylmannose. J. Biol. Chem. 261: 3490-3500, 1986.

Creek, K. E., Rimoldi, D. and De Luca, L. M. Enzymatic synthesis and separation of retinyl phosphate mannose and dolichyl phosphate mannose by anion exchange high performance liquid chromatography. Methods Enzymol. 123: 61-68, 1986.

De Luca, L. M.: Retinoids and cancer: effects on cell adhesion and differentiation. Med. Oncol. 2: 175-180, 1985.

De Luca, L. M. and Creek, K. E.: Vitamin A and the liver. In Popper, H. and Schaffner, F. (Eds.): Progress in Liver Diseases. New York, Grune and Stratton, 1986, Vol. VIII, pp. 81-98.

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De Luca, L. M., Roop, D. and Huang, F. L.: Vitamin A: A key nutrient for the maintenance of epithelial differentiation. Acta Vitaminol. Enzymol. (In Press).

De Luca, L. M., Silverman-Jones, C. S., Rimoldi, D., Warren, C. D. and Creek, K. E.: Retinoids and glycosylation. Chemica Scripta. (In Press).

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Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05177-05 CCTP																												
PERIOD COVERED October 1, 1985 to September 30, 1986																														
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Use of Immunological Techniques to Study Interaction of Carcinogens with DNA																														
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 45%;">M. C. Poirier</td> <td style="width: 20%;">Research Chemist</td> <td style="width: 20%;">LCCTP NCI</td> </tr> <tr> <td colspan="4" style="padding-top: 10px;">Others:</td> </tr> <tr> <td></td> <td>S. H. Yuspa</td> <td>Chief</td> <td>LCCTP NCI</td> </tr> <tr> <td></td> <td>E. Reed</td> <td>Special Assistant for Science</td> <td>DCT NCI</td> </tr> <tr> <td></td> <td>C. Litterst</td> <td>Research Chemist</td> <td>LMCP NCI</td> </tr> <tr> <td></td> <td>R. Ozols</td> <td>Chief</td> <td>MB NCI</td> </tr> <tr> <td></td> <td>T. Hamilton</td> <td>Clinical Associate</td> <td>MB NCI</td> </tr> </table>			PI:	M. C. Poirier	Research Chemist	LCCTP NCI	Others:					S. H. Yuspa	Chief	LCCTP NCI		E. Reed	Special Assistant for Science	DCT NCI		C. Litterst	Research Chemist	LMCP NCI		R. Ozols	Chief	MB NCI		T. Hamilton	Clinical Associate	MB NCI
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COOPERATING UNITS (if any) MIT, Boston, MA (S. Lippard); Univ. of Texas Med. School, Houston, TX (J. M. Hunt); NCTR, Jefferson, AR (F. A. Beland); National Hosp., Oslo, Norway (H. Huitfeldt); CIIT, Res. Triangle Park, NC (J. Swenberg); U. of Iowa, Iowa City, IA (J. Baron); Mt. Sinai Med. Ctr., N.Y., NY (T. Fasy); McArdle, Madison, WI (H. Pitot).																														
LAB/BRANCH Laboratory of Cellular Carcinogenesis and Tumor Promotion																														
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INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892																														
TOTAL MAN-YEARS: 2.5	PROFESSIONAL: 1.0	OTHER: 1.5																												
CHECK APPROPRIATE BOX(ES) X <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews																														
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Antibodies specific for carcinogen-DNA adducts have probed the nature, extent, and consequences of <u>in vitro</u> and <u>in vivo</u> DNA modification. DNAs substituted with 2-acetylaminofluorene (AAF) and <u>cis</u> -diaminedichloroplatinum II (<u>cis</u> -DDP) were analyzed by quantitative immunoassays able to detect about 100 adducts per cell and by immunohistochemical procedures developed to localize adducts <u>in situ</u> . In hepatic DNA of rats fed a carcinogenic dose of AAF for 4 weeks, adduct accumulation reached a plateau at 2-3 weeks. Since adduct removal was biphasic during 4 subsequent weeks on control diet, a computer-derived pharmacokinetic model proposed that adducts are formed into two genomic compartments, one from which they are removed rapidly and another from which they are removed slowly. Studies initiated to identify these two compartments involve adduct analysis in different liver cell types and different chromatin regions including the nuclear matrix. Immunohistochemical localization of AF-DNA adducts in livers of rats fed AAF was shown by fluorescence and microfluorometry to be concentrated primarily in periportal regions. In addition, there were no adducts detectable by immunofluorescence in preneoplastic foci induced by several different protocols. Nucleated peripheral blood cell DNA was obtained from cancer patients at multiple times during courses of <u>cis</u> -DDP therapy, and a total of 254 samples were analyzed. Of these, 23 untreated control samples were negative, and 43% of the 231 samples from patients receiving <u>cis</u> -DDP were positive. Adduct accumulation, in positive patients, occurred as a function of total cumulative dose, suggesting relatively slow adduct removal. Disease response data on 55 patients indicated that individuals with high adduct levels have a high rate of complete response to therapy. Parallel experiments in animal models have demonstrated that the same adduct forms in kidney, gonads, and tumors of rats and mice in direct relation to dose.																														

PROJECT DESCRIPTIONNames, Titles, Laboratory, and Institute Affiliations of Professional Personnel Engaged on this Project:

M. C. Poirier	Research Chemist	LCCTP	NCI
S. H. Yuspa	Chief	LCCTP	NCI
E. Reed	Special Assistant for Science	DCT	NCI
C. Litterst	Research Chemist	LMCP	NCI
R. Ozols	Chief	MB	NCI
T. Hamilton	Clinical Associate	MB	NCI

Objectives:

To develop specific and sensitive quantitative and morphological immunoassays for the investigation of carcinogen-DNA interactions. Studies are directed toward quantitative and qualitative analyses of covalent DNA adduct formation and removal, and localization of adducts at the cellular and subcellular levels. These data are correlated with biological consequences of chemical carcinogen exposure, including cell transformation and tumorigenesis. In the case of the chemotherapeutic agent cis-diamminedichloroplatinum (II) (cis-DDP), biological end points include chemotherapeutic efficacy and short- and long-term toxicity.

Methods Employed:

Both in vivo carcinogen exposure of experimental animals and carcinogen treatment of cultured cells are employed to pursue the objectives. Tissues and cells obtained from individuals environmentally exposed to carcinogens or from patients given cancer chemotherapeutic agents are also utilized. The chemical synthesis of radiolabeled and unlabeled DNA-carcinogen adducts and their purification by column chromatography are currently performed. Isolation of macromolecules for carcinogen binding and repair studies utilize density gradient centrifugation. Antibodies are produced by injection of purified antigens into rabbits. A variety of immunological techniques are employed, including the qualitative procedures of immunofluorescence and immunochemical electron microscopy and the quantitative radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA).

Major Findings:

Interactions of various carcinogens with DNA have been studied in cultured cells, animal organs, and human tissues by a unique immunotechnology pioneered in this section. Rabbit antibodies have been elicited against protein-conjugated carcinogen-nucleoside adducts or methylated-BSA-complexed modified DNAs. The high-affinity antisera obtained have been used to develop radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA) able to detect as little as one adduct in 10^8 nucleotides. The areas of ongoing intensive investigation in this laboratory are as follows: (1) elucidation of mechanisms by which the major rat liver DNA adducts are formed and removed during chronic feeding of a carcinogenic 2-acetylaminofluorene (AAF) regimen and (2) investigation of cis-diamminedichloroplatinum (II) (cis-DDP)-DNA adducts in the nucleated blood cells of testicular and ovarian cancer patients receiving cis-DDP chemotherapy, and in tumor-bearing animal models.

Rabbit antisera specific for guanosin-(8-yl)-2-acetylaminofluorene (G-8-AAF) and guanosin-(8-yl)-2-aminofluorene (G-8-AF) have been utilized in competitive RIAs to monitor profiles of liver DNA adducts during chronic dietary administration of 0.02% AAF to male rats. By feeding nonradioactive and radioactive AAF for varying periods of time, it has been possible to show consistent adduct accumulation both at the beginning and the end of a 4-week period of AAF feeding. In addition, adduct removal and persistence were measured during a subsequent 4 weeks of feeding control diet. These studies culminated in a pharmacokinetic analysis which postulated zero order kinetic adduct formation and first order kinetic adduct removal. Since adduct removal was biphasic, the model postulated two compartments: one susceptible to fast C-8 adduct removal and another from which these adducts are removed much more slowly. The nature of the two differentially-removing compartments has been investigated further in experiments designed to monitor adduct removal (by RIA) in different liver cell types or in different liver chromatin fractions. The former experiments have been performed in collaboration with Dr. J. Swenberg, who separated liver from AAF fed rats into parenchymal, nonparenchymal and bile duct cells by elutriation. Adduct analyses of the different cell types showed that hepatocytes form and remove DNA adducts with biphasic kinetics similar to the whole liver. The nonparenchymal cells form fewer adducts but also show biphasic removal kinetics. The bile duct cells contain few adducts and remove them very slowly; however since these cells account for only about 3% of the total; they are not likely to significantly influence the observed kinetic profiles. The chromatin experiments are being performed in collaboration with Dr. F. Beland and involve fractionation of chromatin DNA by high and low salt extractions to remove portions of DNA at varying distances from the nuclear matrix. These studies are still in the initial states, but have demonstrated that the 5% of total DNA associated with the nuclear matrix contains fewer DNA adducts and removes them more slowly than the other fractions. The kinetics of adduct formation and removal of these chromatin fractions will be investigated further.

In collaboration with Dr. H. Huitfeldt, immunohistochemical localization of DNA adducts in livers of male Fischer rats chronically fed AAF has been observed in nuclei of liver frozen sections stained by an anti-G-8-AF. The overall pattern of adduct formation was nonuniform, with the highest adduct concentration in the periportal areas and a decreasing gradient through the midzonal to the centrilobular areas. This pattern did not change during a month of chronic AAF feeding or during a subsequent month on control diet (even though the intensity of fluorescence changed), suggesting that differences in lobular localization are not responsible for the biphasic kinetics of adduct removal. The lobular localization obtained during 28 days of AAF feeding was confirmed in a quantitative fashion by microfluorometry, performed in collaboration with Dr. J. Baron. These studies showed that during chronic AAF feeding midzonal hepatocytes contained 80% of the fluorescence intensity exhibited by hepatocytes, and the bile duct epithelial cells contained only about 20% of the hepatocyte fluorescence. The microfluorometric profiles of adduct accumulation were similar to the whole-liver profile of adduct accumulation determined by RIA. Immunohistochemical studies were further utilized to examine preneoplastic enzyme-altered foci for the presence of AAF-DNA adducts. In these studies, the induction of foci by a variety of procedures was followed by 6 days of AAF feeding just prior to sacrifice. The localization of areas positive for γ GT or

negative for G-6-Pase or ATPase was determined, in collaboration with Dr. H. Pitot, by computer analysis. The same areas were identified by immunofluorescence and all shown to be negative for the dG-8-AF adduct formation.

cis-DDP is a remarkably potent chemotherapeutic agent which induces the formation of intrastrand N⁷-deoxy(GpG)- and N⁷-deoxy(GpA)-diammineplatinum adducts as a major fraction of total platinum bound to DNA. In collaboration with Dr. S. Lippard, we have elicited a polyclonal antibody specific for these adducts and developed an ELISA capable of detecting 25 attomoles of adduct/ μ g DNA. Using the anti-cis-DDP-DNA-ELISA, we have analyzed DNA extracted from nucleated peripheral blood cells (buffy coat) of controls and testicular and ovarian cancer patients at multiple times during cis-DDP treatment. Of these, all 23 samples from untreated controls were never positive, and 43% of the 231 samples from cis-DDP-treated patients were positive. Patients on their first course of chemotherapy receiving cis-DDP on 21- or 28-day cycles (5 days of drug infusion followed by 2 or 3 drug-free weeks) accumulated DNA adducts as a function of dose and increasing cycle number. An analysis of disease response data for 55 patients showed that individuals forming high levels of cis-DDP adducts (>200 attom/ μ g DNA) are more likely to undergo complete remission than those forming fewer adducts or no adducts at all. ELISA analysis of human tissues obtained at autopsy (by Dr. T. Fasy) from cis-DDP-treated cancer patients showed that these adducts can persist in kidney DNA for months subsequent to the last treatment. This implies that the adduct removal processes work slowly.

Selected tissues of rats and mice were examined following intraperitoneal injections (IP) of cis-DDP in collaboration with Dr. C. Litterst. Tissues known to be targets for cis-DDP therapy or toxicity, such as kidney, gonad, and tumor, were chosen. A dose response was seen in kidneys and gonads of males and females, although adducts were higher in kidneys than in gonads and higher in males than in females. Castrated animals were used to investigate the role of sex hormones in adduct formation in male and female rats. Since the castrated animals formed kidney-DNA adducts similar to sham-operated controls, sex hormones were considered not to be a major modulating influence. Studies are being performed in collaboration with Dr. R. Ozols and Dr. T. Hamilton to investigate cis-DDP-DNA adduct formation in nude mice bearing an intraperitoneal human ovarian carcinoma implant. cis-DDP-DNA adducts were monitored in kidney, solid tumor and ascites 4 h following an IP drug injection, and all three tissues exhibited similar adduct quantities.

Publications:

Huitfeldt, H. S., Spangler, E. F., Hunt, J. M. and Poirier, M. C.: Immunohistochemical localization of DNA adducts in rat liver tissue and phenotypically-altered foci during oral administration of 2-acetylaminofluorene. Carcinogenesis 7: 123-129, 1986.

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Perera, F. P., Santella, R. M. and Poirier, M. C.: Potential methods to monitor human populations exposed to carcinogens: Carcinogen-DNA binding as an example. In Hoel, D., Merrill, R., Perera, R. (Eds.): Banbury Report 19. Banbury Conference On Risk Quantitation and Regulatory Policy. New York, Cold Spring Harbor Laboratory, 1985, pp. 211-229.

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Poirier, M. C., Reed, E., Zwelling, L. A., Ozols, R. F., Litterst, C. L. and Yuspa, S.H.: The use of polyclonal antibodies to quantitate cisdiammine-dichloroplatinum (II)-DNA adducts in cancer patients and animal models. Environ. Health Perspect. 62: 89-94, 1985.

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Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05178-05 CCTP
PERIOD COVERED October 1, 1985, to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Cellular and Tissue Determinants of Susceptibility to Chemical Carcinogenesis		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	J. E. Strickland	Research Chemist LCCTP NCI
Others:	S. H. Yuspa	Chief LCCTP NCI
	H. Hennings	Research Chemist LCCTP NCI
	J. Harper	Staff Fellow LCCTP NCI
COOPERATING UNITS (if any) Microbiological Associates, Bethesda, Maryland (E. F. Spangler)		
LAB/BRANCH Laboratory of Cellular Carcinogenesis and Tumor Promotion		
SECTION In Vitro Pathogenesis Section		
INSTITUTE AND LOCATION NIH, NCI, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.4	2.4	
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> <u>In vivo</u> studies have shown that the SENCAR mouse is unusually sensitive to skin carcinogenesis by initiation and promotion while the Balb/c mouse is resistant. We have developed a technique of grafting epidermal and dermal cells to athymic nude mice in order to form a reconstituted skin. Cell line 308RB was previously developed in this laboratory by initiation of adult Balb/c mouse skin <u>in vivo</u> with 7, 12-dimethylbenz[a]anthracene (DMBA), culture of the epidermal cells, and selection for cells resistant to Ca²⁺-induced terminal differentiation, a property of initiated cells. Using the grafting technique, we have shown that line 308RB forms benign squamous papillomas, confirming that it is an initiated, nonmalignant cell line. We have also developed cell lines from papillomas produced on both SENCAR (SP lines) and Balb/c (BP lines) mice <u>in vivo</u> by initiation with DMBA and promotion with 12-O-tetradecanoylphorbol-13-acetate (TPA). When grafted to nude mice, these lines produce papillomas similar to those formed by line 308RB. Line SP-1, the most extensively studied to date, produces papillomas whose size increases as the number of cells grafted increases from 16,000 to 1,600,000. Addition of normal primary epidermal cells at the time of grafting suppresses papilloma formation by SP-1 cells in proportion to the number of normal cells added. The development of these cell lines provides a means for controlled study of the suppression of Balb/c and SENCAR papilloma development by homologous and heterologous normal primary epidermal cells and the overcoming of this suppression in response to tumor promotion. Furthermore, these cell lines are a useful model for study of the conversion of benign to malignant tumor cells. </p>		

PROJECT DESCRIPTION

Names, Titles, Laboratory, and Institute Affiliations of Professional Personnel Engaged on this Project:

James E. Strickland	Research Chemist	LCCTP	NCI
Stuart H. Yuspa	Chief	LCCTP	NCI
Henry Hennings	Research Chemist	LCCTP	NCI
John Harper	Staff Fellow	LCCTP	NCI

Objectives:

To elucidate the cellular and molecular mechanisms of enhanced sensitivity to carcinogenesis in genetically-derived susceptible mouse strains.

Methods Employed:

The SENCAR mouse was developed by a selective breeding protocol for enhanced susceptibility to skin carcinogenesis by initiation and promotion. Comparisons are made to Balb/c mice as a representative resistant strain in vivo and in vitro studies. The epidermis can be separated from the dermis by flotation of the skin, dermis side down, on a solution containing trypsin. The separated epidermal cells are cultured in medium containing Ca^{2+} levels <0.1 mM to induce terminal differentiation. Initiated cells can be selected from an excess of normal cells on the basis of the resistance of the former to Ca^{2+} -induced terminal differentiation. Papilloma cell lines were developed from papillomas produced on adult SENCAR and Balb/c mouse skin by initiation with DMBA and promotion with TPA. Papillomas were removed, minced, and cells were dissociated by treatment with collagenase, followed by trypsin, and cells were cultured in medium with low $[\text{Ca}^{2+}]$. A grafting system has been developed in athymic nude mice which can produce normal skin from cultured primary epidermal and dermal cells of newborn mice. A vascularized graft bed is produced by subcutaneous implantation of a glass disk (3 mm thick X 17 mm diam.) one week prior to grafting. Cultured cells are released from flasks or dishes by treatment with trypsin, and a mixture of epidermal and dermal cells is centrifuged and the cell pellet is applied to the graft bed within a silicone chamber which separates the graft from the host skin. After one week, the chamber is removed and the graft is bandaged for an additional week, following which the bandage is removed, and the wound allowed to heal. Grafts are examined both grossly and histologically at sacrifice.

Major Findings:

The grafting system has been used to test a number of presumed initiated cell lines derived previously in this laboratory. Many of these lines, previously found to be nontumorigenic by subcutaneous injection or implantation on plastic liners, have formed carcinomas in skin grafts. Thus, we believe that the grafting system is more sensitive for testing tumorigenicity of cells derived from the epidermis than is subcutaneous injection. Only one initiated cell line so far tested, line 308RB, has formed benign squamous papillomas in skin grafts. This line was derived from adult Balb/c mouse epidermis, initiated in vivo

with 7,12-dimethylbenz[a]anthracene (DMBA) and selected in culture for resistance to Ca^{2+} -induced terminal differentiation, an inferred property of initiated cells. A considerable amount of study of the properties of line 308RB in culture has been done in recent years, so this line will be useful as a model for examining the molecular nature of the conversion of benign papillomas to carcinomas.

We have recently derived cell lines from papillomas produced on mice in vivo by initiation and promotion protocols. The most extensively studied of these is line SP-1, developed from papillomas on SENCAR mice initiated with DMBA and promoted with 12-O-tetradecanoyl-phorbol-13-acetate (TPA). Several lines (designated BP) have also been developed from papillomas produced on Balb/c mice by a similar protocol. These lines form benign squamous papillomas when grafted onto nude mice as described above. The SP and BP lines, along with line 308RB, are being used both in culture and in vivo to study the biology of tumor promotion and conversion. Conversion studies using transfection of the SP, BP, and 308RB cells with DNA from rat epidermal carcinomas and assay of the resultant transfectants for formation of carcinomas in the graft system will allow identification of the transfected sequences by means of rat-specific probes. Lines SP-1 and 308RB have already been shown to have an activated oncogene, presumably ras, by transfection of their DNA into NIH 3T3 test cells. Restriction enzyme analysis is underway to determine with certainty whether this oncogene is indeed ras. Other studies in this laboratory have demonstrated that primary mouse epidermal cells can be initiated when an activated ras^{Ha} is inserted in them by means of an engineered virus incapable of productive replication. Such cells are then capable of forming benign squamous papillomas in skin grafts to nude mice. Thus, it appears that not only is the ras oncogene able to produce initiated cells from normal epidermal cells, but activation of the ras gene is also accomplished by chemical initiation and promotion protocols with DMBA and TPA in vivo. It is also clear that other non-ras mechanisms of initiation exist since we have shown that several cell lines, derived previously from papillomas but now malignant, do not have an activated ras gene.

In further grafting studies with line SP-1, we have shown that papillomas can be obtained with as few as 16,000 SP-1 cells per graft and that larger papillomas are obtained as the number of cells is increased to 160,000 and to 1,600,000. We have also demonstrated that the addition of normal primary epidermal cells to SP-1 cells at the time of grafting results in suppression of papilloma formation, with the degree of suppression proportional to the number of normal cells added. The system will now permit studies in which suppression is complete, and tumor promoters such as TPA will be used to determine whether promotion will be able to overcome the suppression and lead to papilloma formation, as we would predict. We can also compare the relative efficiency of Balb/c and SENCAR primary epidermal cells in suppressing formation of papillomas by SP, BP, and 308RB cells to determine whether cells of one strain are more effective in suppression or more responsive to promotion in overcoming the suppression.

Recent experiments indicate that SP-1 cells, even though they apparently already have an activated ras gene, can be converted to malignancy by insertion of an activated ras gene in a nonproducer virus engineered with a promoter to induce

high levels of expression of the ras p21 protein kinase gene product. These results are important because they demonstrate that SP-1 does not form "dead-end" papillomas, which are not on the pathway to malignancy and therefore no more likely to become carcinomas than are normal cells. Furthermore, these results suggest that ras gene expression alone, at sufficiently high levels, can result in malignancy. We cannot, of course, rule out activation of other genes, which may play a role in the carcinogenesis process, as a result of the addition of the activated ras gene to the cells.

Publications:

Lichti, U., Strickland, J. E., Jeng, A. Y. and Blumberg, P. M.: Phospholipase C mimics 12-O-tetradecanoylphorbol-13-acetate in the induction of ornithine decarboxylase and other biochemical changes in mouse epidermal keratinocytes. In Selmecki, L., Brosnan, M. E. and Seiler, N. (Eds.): Recent Progress in Polyamine Research. Budapest, Adademiai Kiado, 1985, pp. 413-422.

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Strickland, J. E., Jetten, A. M., Kawamura, H. and Yuspa, S. H.: Interaction of epidermal growth factor (EGF) with basal and differentiating mouse keratinocytes. In Murakami, H., Yamane, I., Barnes, D. W., Mather, J. P., Hiyashi, I., and Sato, G. H. (Eds.): Growth and Differentiation of Cells in Defined Environment. Berlin, Springer-Verlag, 1985, pp. 419-424.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05270-05 CCTP
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Molecular Mechanism of Action of Phorbol Ester Tumor Promoters		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Peter M. Blumberg	Research Chemist LCCTP NCI
Others:	A. Y. Jeng	Expert LCCTP NCI
	M. Dell'Aquila	Staff Fellow LCCTP NCI
	B. Warren	Guest Researcher LCCTP NCI
	T. Nakadate	Visiting Fellow LCCTP NCI
	G. Pasti	Visiting Fellow LCCTP NCI
	T. Sako	Visiting Fellow LCCTP NCI
COOPERATING UNITS (if any) Boston Univ. School of Med., Boston, MA (A. I. Tauber, J. Cox); Stanford Univ. Palo Alto, CA (P. Wender, K. Koehler); Arizona State Univ., Tempe, AZ (G. R. Pettit)		
LAB/BRANCH Laboratory of Cellular Carcinogenesis and Tumor Promotion		
SECTION Molecular Mechanisms of Tumor Promotion Section		
INSTITUTE AND LOCATION NIH, NCI, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7.1	6.1	1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> The efforts of the Molecular Mechanisms of Tumor Promotion Section are directed at understanding the early events in the interaction of phorbol ester tumor promoters with cells and tissues. Particular attention is being devoted to the analysis of the major phorbol ester receptor, protein kinase C. Most of the evidence for the role of protein kinase C in phorbol ester action is indirect. Its involvement in the mitogenic response of Swiss 3T3 cells was demonstrated directly by microinjection, using Swiss 3T3 cells down-regulated by chronic phorbol ester treatment. Emerging evidence suggests prominent proteolytic processing of protein kinase C. The catalytically active fragment of the enzyme was prepared by tryptic digestion and was characterized. Phospholipids regulated its activity in a pH- and substrate-dependent fashion, distinct from the activation of the intact enzyme by the interaction of phospholipids at the regulatory domain. The regulatory domain, being devoid of enzymatic activity and thus a pseudo-receptor, was also generated and is now being characterized. Analysis of modulators of protein kinase C other than the phorbol esters afford unique insights into its function. Bryostatins, macrocyclic lactones, activate protein kinase C and compete for phorbol ester binding. Paradoxically, they blocked phorbol ester action in two differentiating systems (HL-60 promyelocytic leukemia cells and Friend erythroleukemia cells) and blocked some but not other responses in keratinocytes. In HL-60 cells, the bryostatins induced a similar but distinct pattern of phosphorylation. Diglycerides are the postulated endogenous analogs of the phorbol esters. In support of this hypothesis, treatment of mouse keratinocytes with bromooctanoic acid, which blocks triglyceride formation and causes diglyceride accumulation, induced effects similar to the phorbol esters. Bromooctanoic acid may provide a tool for assessing the tumor promoting activity of the diglycerides. Modeling studies comparing different classes of protein kinase C activators indicated putative common features. Novel structures incorporating these features inhibited phorbol ester binding and induced typical phorbol ester responses such as inhibition of EGF binding or phosphorylation of a 40 kd platelet protein. </p>		

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

P. M. Blumberg	Research Chemist	LCCTP	NCI
A. Y. Jeng	Expert	LCCTP	NCI
M. Dell'Aquila	Staff Fellow	LCCTP	NCI
B. Warren	Guest Researcher	LCCTP	NCI
T. Nakadate	Visiting Fellow	LCCTP	NCI
G. Pasti	Visiting Fellow	LCCTP	NCI
T. Sako	Visiting Fellow	LCCTP	NCI
S. Yuspa	Chief	LCCTP	NCI
H. Hennings	Research Chemist	LCCTP	NCI
S. Aaronson	Chief	LCMB	NCI
J. Lacal	Visiting Fellow	LCMB	NCI

Objectives:

The early events in the interaction of the phorbol esters with cells and tissues are being characterized. Specific aims are as follows: 1) Analysis of the functional domains of protein kinase C in vitro; 2) determination of the biological activities of intact protein kinase C and its functional domains upon micro-injection back into cells; 3) elucidation of mechanistic differences between different classes of protein kinase C activators; 4) understanding of the role of diglycerides and protein kinase C in keratinocyte differentiation and tumor promotion; 5) identification of targets for the phorbol esters and related diterpenes in addition to protein kinase C. The major phorbol ester receptor, protein kinase C, is postulated to mediate one of the two pathways activated by a large class of hormones for which receptor occupancy is associated with rapid phosphatidylinositol turnover. Several oncogenes may also function, in part, through this pathway. Understanding of the mechanisms of endogenous modulation of the phorbol ester receptors may thus provide insights into both basic biochemical mechanisms and the process of human carcinogenesis as well as to identify biochemical steps suitable for intervention.

Methods Employed:

This Section uses a wide range of techniques to pursue the above aims. Phorbol and derivatives are isolated from natural sources. Semisynthetic derivatives for affinity labeling, structure-activity analysis, and binding studies are prepared and radioactively labeled as necessary. Binding studies are carried out using the ligands and methodology developed by us. Analysis of receptors utilizes both photoaffinity labeling and standard biochemical membrane methodology. The systems analyzed are chosen as optimal for the specific questions being examined. Brain homogenates, because of their richness in receptors, are being used for receptor purification and biochemical analysis. Mouse skin and cultured keratinocytes are being used to dissect subclasses of receptors and to study the relation between protein kinase C, cell differentiation, and tumor

promotion. Intact cells, HL-60 promyelocytic leukemia cells and human neutrophils, are being utilized to elucidate the coupling between receptor occupancy and biological responses. Microinjection of receptor domains is employed to clarify their cellular function. Importance is placed upon the ability to relate the answers obtained to the biological system of mouse skin promotion and to coordinate, effectively, exploitation of the systems being studied by the other Sections of the Laboratory.

Major Findings:

A central issue in understanding the mechanism of phorbol ester action is the discrepancy between a single major receptor, protein kinase C, and both biological and binding data indicative of heterogeneity in phorbol ester action. A mechanism which has received increasing interest is that of proteolytic processing of protein kinase C. As first reported by Nishizuka, digestion by Ca^{++} -activated protease generates an active fragment which no longer requires Ca^{++} and anionic phospholipids, essential cofactors for the intact enzyme. Phorbol ester treatment in intact cells appears to accelerate the rate of fragment formation. To explore the properties and biological significance of the catalytically active fragment, we established conditions for its generation by tryptic digestion of the purified enzyme and its subsequent isolation. A surprising finding during its characterization was that the enzymatic activity of the active fragment, although it did not require phospholipids, was still subject to modulation by them. The effect of phospholipids was more marked at acidic pH and depended on the substrate being used. Activity on histone H1 and myelin basic protein was subject to modulation; activity on fibronogen was not. These results suggest that lipids may regulate intact protein kinase C not only through the previously characterized interaction at the regulatory domain but also through an enzyme-phospholipid-substrate ternary complex.

Analysis of the kinetics of formation of the catalytic fragment revealed that phorbol ester binding activity remained stable under digestion conditions which caused complete loss of enzymatic activity. Conditions for stabilization of the binding domain have been established. Current efforts are directed at its isolation and characterization.

Most of the evidence for the participation of the intact protein kinase C in phorbol ester responses is indirect. No evidence is available regarding the possible role of the catalytic fragment. We have begun to use microinjection to explore these questions. In Swiss 3T3 cells, phorbol ester treatment for 48 hours causes loss of protein kinase C and of mitogenic response to the phorbol esters. Microinjection of homogeneous protein kinase C into the pretreated cells (collaborative experiments with J. C. Lacal and S. Aaronson) restored phorbol ester responsiveness. Response was dependent on the presence of phorbol ester and was not seen in cells injected with carrier protein.

A second strategy for studying heterogeneity is to compare the behavior of structurally different classes of protein kinase C activators. Bryostatin, a recently described macrocyclic lactone isolated from a marine bryozoan, has proven to be of particular interest. Although it inhibits phorbol ester binding and activates protein kinase C, bryostatin functionally antagonizes some but not

other phorbol ester responses. In collaborative experiments with G. R. Pettit, we have shown that bryostatin blocks both phorbol ester induced adhesion and growth inhibition in HL-60 cells. This latter effect strongly argues against the antagonistic action of the bryostatins resulting from generalized toxicity. Comparison of the patterns of phosphorylation in HL-60 in response to phorbol esters and bryostatins indicate that the bryostatins induce all of the same changes as do the phorbol esters, but also cause the phosphorylation of an additional substrate. Competition experiments, like the phosphorylation studies, suggest that the antagonistic effect of bryostatin is through interaction at a target other than protein kinase C. Antagonism of phorbol ester actions is observed in two other systems. In Friend erythroleukemia cells, bryostatin alone has no effect, but it blocks response to the phorbol esters. In this case, phorbol esters inhibit the induction of differentiation by DMSO or hexamethylene bisacetamide. Bryostatin restores the differentiation response. In mouse keratinocytes, bryostatin blocks the phorbol ester-induced morphological change and induction of transglutaminase. By itself, it stimulates ornithine decarboxylase, however. It thus appears to favor the proliferative, but not the differentiative, pathway in these cells. In mouse skin, bryostatin is hyperplasiagenic and is currently being tested for its activity either as a tumor promoter or as an inhibitor of phorbol ester tumor promotion (collaborative studies with H. Hennings and S. Yuspa).

Binding studies suggested that diglycerides might be the endogenous analogs of the phorbol esters. As part of our effort to demonstrate that the mechanism of inhibition of phorbol ester binding was competitive, we analyzed the kinetics of the phorbol ester off-rate from protein kinase C as a function of diacylglycerol concentration. The data supported a competitive mechanism. If diglycerides are endogenous phorbol ester analogs, elevated diglyceride levels might be tumor promoting. The usual methods for elevating diglyceride levels, use of soluble diglycerides or phospholipase C treatment, are unsuitable for treatment of animals. Alpha-bromooctanoic acid blocks triglyceride synthesis, thereby causing accumulation of diglyceride. Alpha-bromooctanoic acid stimulated ornithine decarboxylase and inhibited epidermal growth factor binding in mouse keratinocytes (collaborative studies with S. Yuspa) similarly to the phorbol esters. It likewise induced hyperplasia in mouse skin; assay of its tumor promoting activity is in progress (collaborative studies with H. Hennings and S. Yuspa).

Antagonists of the phorbol esters would be of considerable potential interest both as anti-promoters and as modulators of cellular effectors which function through phosphatidylinositol turnover. Computer comparison by P. Wender (Stanford University) of the phorbol esters and of the indole alkaloids suggested positional homology between the hydroxyl groups at C4, 9, and 20 of phorbol and corresponding groups on the indole alkaloids. Since the phorbol esters are complicated structures, simple active ring systems would be of considerable utility for probing structure-function relations at the phorbol binding site. Two classes of such simplified ring systems substituted to be isosteric with the putative three critical hydroxyl groups of phorbol were prepared. They all inhibited phorbol ester binding, albeit with potencies similar to the diglycerides and lower than those of the phorbol esters. For individual derivatives, in vivo responses typical of the phorbol esters, such as inhibition

of epidermal growth factor binding in 3T3 cells or stimulation of phosphorylation in platelets of a 40 kd protein kinase C substrate, were obtained. The activity of these compounds supports the validity of the modeling approach. More sophisticated analogs are now being prepared in an effort to increase potency.

Publications:

Blumberg, P. M., Jeng, A. Y., Konig, B., Sharkey, N. A., Leach, K. L. and Jaken, S.: Phorbol esters as probes of the regulatory site on protein kinase C. In Imura, H., Goto, T., Murachi, T. and Nakajima, T. (Eds.): Natural Products and Biological Activities. New York, Elsevier, 1986, pp. 167-178.

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Dunn, J. A., Jeng, A. Y., Yuspa, S. H. and Blumberg, P. M.: Heterogeneity of [³H]phorbol 12,13-dibutyrate binding in primary mouse keratinocytes at different stages of maturation. Cancer Res. 45: 5540-5546, 1985.

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Konig, B., DiNitto, P. A., and Blumberg, P. M.: Stoichiometric binding of diacylglycerol to the phorbol ester receptors. J. Cell Biochem. 29: 37-44, 1985.

Lichti, U., Strickland, J. E., Jeng, A. Y. and Blumberg, P. M.: Phospholipase C mimics 12-O-tetradecanoylphorbol 13-acetate in the induction of ornithine decarboxylase and other biochemical changes in mouse epidermal keratinocytes. In Selmecki, L., Brosnan, M. E. and Seiler, N. (Eds.): Recent Progress in Polyamine Research. Oxford, Pergamon Press, 1985, pp. 413-422.

Sharkey, N. A. and Blumberg, P. M.: Comparison of the activity of phorbol 12-myristate 13-acetate and the diglyceride glycerol 1-myristate 2-acetate. Carcinogenesis 7: 677-679, 1986.

Sharkey, N. A. and Blumberg, P. M.: Kinetic evidence that 1,2-diolein inhibits phorbol ester binding to protein kinase C via a competitive mechanism. Biochem. Biophys. Res. Commun., 133: 1051-1056, 1985.

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Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05445-02 CCTP
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Molecular Regulation of Epidermal Specific Differentiation Products		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Dennis R. Roop	Senior Staff Fellow LCCTP NCI
Others:	S. H. Yuspa	Chief LCCTP NCI
	J. Harper	Staff Fellow LCCTP NCI
	L. De Luca	Research Chemist LCCTP NCI
	P. Steinert	Visiting Scientist DB NCI
	J. Stanley	Medical Officer DB NCI
	S. Chung	Senior Staff Fellow LEC NCI
	G. Peck	Senior Investigator DB NCI
COOPERATING UNITS (if any) Microbiological Assoc., Bethesda, MD (E. F. Spangler); Univ. of Munich (Thomas Krieg).		
LAB/BRANCH Laboratory of Cellular Carcinogenesis and Tumor Promotion		
SECTION In Vitro Pathogenesis Section		
INSTITUTE AND LOCATION NIH, NCI, DCE Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4	3	1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> cDNA clones corresponding to the major keratins expressed in mouse epidermis have been isolated and characterized. Using a combination of <u>in situ</u> hybridization with RNA probes, which are specific for individual keratin mRNAs, and indirect immunofluorescence with monospecific antisera, which were elicited with synthetic peptides corresponding to unique sequences within each keratin subunit, it is possible to show that these keratin genes belong to at least three subsets: those expressed predominantly in the proliferating basal layer of the epidermis, those expressed predominantly in the differentiated suprabasal layers of the epidermis, and those only expressed under hyperproliferative conditions such as in benign and malignant epidermal tumors, hyperplasia induced by the tumor promoter TPA and squamous metaplasia induced by various means in hamster trachea. Genes representing each subset have been isolated and sequenced. Various strategies have been employed to identify sequences regulating the expression of these genes, including vector constructs using different regions of the genomic clones to drive expression of the neomycin-resistance gene and the chloramphenicol acetyl transferase gene and the production of transgenic mice containing genomic fragments encoding human keratins. The <u>in situ</u> hybridization technique has been used to confirm previous results obtained with monospecific antisera which demonstrated that malignant epidermal tumors could be distinguished from benign tumors due to their failure to express the differentiation-associated keratins. The <u>in situ</u> hybridization technique has also been used to study the expression of keratin genes in human biopsies of epidermal tumors and other skin disorders. In addition, this technique has been used to localize transcripts of other genes that are differentially expressed in the epidermis such as genes coding for a thiol proteinase inhibitor, a cornified envelope protein, an activated Harvey <u>ras</u> oncogene and a keratin filament aggregating protein. </p>		

PROJECT DESCRIPTION

Names, Titles, Laboratory, and Institute Affiliations of Professional Personnel Engaged in this Project:

Dennis R. Roop	Senior Staff Fellow	LCCTP	NCI
S. H. Yuspa	Chief	LCCTP	NCI
J. Harper	Staff Fellow	LCCTP	NCI
L. De Luca	Research Chemist	LCCTP	NCI
P. Steinert	Visiting Scientist	DB	NCI
J. Stanley	Medical Officer	DB	NCI
S. Chung	Senior Staff Fellow	LEC	NCI
G. Peck	Senior Investigator	DB	NCI

Objective:

To isolate and characterize the genes coding for the major differentiation products of epidermal cells, the keratins. To study the expression of these genes during normal differentiation and during various stages of carcinogenesis.

Methods Employed:

The isolation of keratin cDNA clones is accomplished by the purification of epidermal mRNA, reverse transcription and cloning of double-stranded cDNA in the plasmid pBR322. The cDNA clones are characterized by hybridization-selection assays and by direct DNA sequence analysis. Keratin genes are isolated by screening genomic libraries with nick-translated cDNAs and characterized by restriction endonuclease digestion and direct DNA sequence analysis. The expression of specific keratin genes is monitored by RNA blot analysis and quantitated by slot-blot analysis. Transcripts for individual keratin genes are detected in histological sections of skin by *in situ* hybridization with ³⁵S labeled RNA probes. Monospecific antisera are produced with synthetic peptides corresponding to unique sequences within keratin subunits. The antisera are used to monitor normal and abnormal expression of keratin polypeptides by immunofluorescent staining, immunoblotting, and immunoprecipitation.

Major Findings:

The production of very specific antibodies with synthetic peptides that permit the detection of individual keratin subunits and the development of an *in situ* hybridization technique that allows the localization of keratin gene transcripts within cells in different layers of the epidermis have revealed the high degree of regulation that exists for these genes in the epidermis. One subset of keratin genes referred to as proliferation-associated is only expressed in the basal layer. After cells commit to terminal differentiation and migrate away from the basement membrane, these keratin genes are turned off. In addition, there appears to be regulation at the level of RNA stability since few transcripts are detected in the differentiated layers. Another set of keratin genes (differentiation-associated) is expressed concomitantly with the decision to terminally differentiate and this appears to occur in some cells prior to migration away from the basement membrane since transcripts and translation products can be detected in some cells still in contact with the basement membrane.

In order to elucidate the mechanism regulating the expression for these keratin genes, members of each subset have been isolated and sequenced. In initial attempts to define sequences which regulate the expression of these genes, flanking sequences of one of the differentiation-associated genes have been inserted into vectors containing the neomycin-resistance gene and the chloramphenicol acetyl transferase gene. To date, expression of neither vector has been observed. This may be due to the absence of regulatory sequences in the flanking sequences inserted or to difficulties in inducing terminal differentiation in the cell cultures employed. In an attempt to overcome both of these problems, transgenic mice are being produced in collaboration with Dr. Su Chung (LEC) which will contain a differentiation-associated human keratin gene. Specific probes are available to distinguish expression of the human keratin gene from the endogenous mouse gene.

In addition to the keratin genes which belong to the proliferation-associated or differentiation-associated subsets, we have isolated another keratin gene and shown by in situ hybridization and indirect immunofluorescence with a monospecific antibody that this keratin gene is not expressed in normal epidermis but is expressed under hyperproliferative conditions such as in primary epidermal cell culture, benign and malignant epidermal tumors, epidermal hyperplasia induced by the tumor promoter TPA, and squamous metaplasia induced in hamster trachea during vitamin A deficiency or exposure to chemical carcinogens (studied in collaboration with Dr. Luigi De Luca [LCCTP]). Since the ability to specifically detect expression of this keratin gene may be of diagnostic value, we are currently attempting to isolate the human gene corresponding to this keratin and produce monospecific antibodies.

Previous results, obtained with monospecific antisera, indicated that benign epidermal tumors could be distinguished from malignant tumors on the basis of a lack of expression of the differentiation-associated keratins in the malignant tumors. This observation has been confirmed by in situ hybridization studies and shown to be due to the absence of transcripts of these genes and not a translational defect. These results, in combination with those obtained for the keratin gene only, expressed under hyperproliferative conditions, demonstrates the usefulness of the keratins as markers to follow tumor progression. Pre-malignant lesions can be detected at very early stages with probes for the hyperproliferative keratin and probes for the differentiation-associated keratins allow discrimination between benign and malignant tumors.

In collaboration with Dr. Thomas Krieg (University of Munich), Dr. Gary Peck (Dermatology Branch, NCI) and Dr. Peter Steinert (Dermatology Branch, NCI), a project has been initiated to study keratin gene expression in human malignancies of the skin and in other skin disorders where keratinization defects are suspect. Gene expression at the transcriptional level is being detected by in situ hybridization with RNA probes specific for the human keratins and expression at the translational level is being determined by indirect immunofluorescence with antisera that are monospecific for the human keratins. Preliminary findings suggest that indifference to normal epidermis where the expression of the proliferation-associated keratin genes is suppressed after cells migrate away from the basement membrane, hyperproliferative disorders of the epidermis exhibit inappropriate expression of the proliferation-associated keratin genes in layers far away from the basement membrane. In addition, as

observed in mouse epidermal tumors, some of the malignant human tumors fail to express the differentiation-associated keratins and this may prove to be useful diagnostically.

The development of the in situ hybridization technique has not only proven valuable in visualizing the expression of keratin genes but also other genes that are differentially expressed in the epidermis. Three of these genes are described in project Z01CP04504-14 CCTP and include: a gene encoding a low molecular weight thiol proteinase inhibitor, expressed in the basal and spinous layers; a gene encoding a putative cornified envelope protein, expressed in the granular layer; an activated Harvey ras oncogene introduced into primary epidermal cells by a defective retroviral vector, expressed in the basilar portions of benign tumors. A fourth gene, studied in collaboration with Dr. Peter Steinert (Dermatology Branch, NCI), encodes filaggrin (a histidine-rich protein responsible for aggregating keratin filaments into the dense filament-matrix complex of the stratum corneum) and is highly expressed in the granular layer of the epidermis.

Publications:

Banks-Schlegel, S. P. and Roop, D. R.: Intermediate filaments. In Trump, B. F. and Mergner, W. J. (Eds.): Mechanisms of Cell Damage and Cell Death. Boca Raton, CRC Press, Inc. (In Press).

Hawley-Nelson, P., Roop, D. R., Cheng, C. K. and Yuspa, S. H.: Regulated synthesis of low molecular weight antigen in keratinocyte cell cultures. J. Invest. Dermatol. (In Press).

Huang, F. L., Roop, D. R. and De Luca, L. M.: Vitamin A deficiency and keratin biosynthesis in cultured hamster trachea. In Vitro 22: 223-230, 1986.

Roop, D. R.: Regulation of keratin gene expression during differentiation of epidermal and vaginal epithelial cells. In Mascona, A. A. and Monroy, A. (Eds.): Current Topics in Developmental Biology. Orlando, Academic Press. (In Press).

Roop, D. R., Cheng, C. K., Toftgard, R., Stanley, J. R., Steinert, P. M. and Yuspa, S. H.: The use of cDNA clones and monospecific antibodies as probes to monitor keratin gene express. Ann. N.Y. Acad. Sci. 455: 426-435, 1985.

Roop, D. R. and Steinert, P. M.: The structure and evolution of intermediate filament genes. In Shay, J. W. (Ed.): Cell and Molecular Biology of the Cytoskeleton. New York, Plenum Press, 1986, 69-83.

Steinert, P. M., Idler, W. W., Zhao, X.-M., Johnson, L. D., Parry, D. A. D., Steven, A. C., Roop, D. R.: Structural and functional implications of amino acid sequences of keratin intermediate filament subunits. Ann. N.Y. Acad. Sci. 455: 451-461, 1985.

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Patents:

None

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ANNUAL REPORT OF
THE LABORATORY OF CHEMOPREVENTION
CHEMICAL AND PHYSICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1985 through September 30, 1986

The problem of the isolation, characterization, and biological role of transforming polypeptide growth factors (TGFs) continues to be the major focus of our Laboratory. Previously, we had shown that TGFs can be isolated from a variety of epithelial and mesenchymal tumors of murine, chicken, and human origin, caused either by chemicals or viruses, or of spontaneous origin. All of these TGFs are acid-stable, low molecular weight peptides, that are the subject of current attempts at amino acid sequencing. New methods to achieve purification have been developed in our Laboratory, and we have completed the total purification of TGF-beta from three non-neoplastic tissues. These tissues are human placenta, human blood platelets, and bovine kidney. The experimental use of TGF-beta in wound healing has been a finding of major importance and has provided a great deal of encouragement to proceed further with the entire problem of the molecular biology and molecular genetics of these growth factors. Finally, we are now involved in a major attempt to integrate studies of retinoids into our current program of studies on peptide growth factors.

The present activities of the Laboratory are devoted almost exclusively to studying the chemistry and biology of both type alpha and type beta transforming growth factors. These studies include the interactions of these peptide growth factors with the genetic apparatus of the cell, particularly oncogenes, as well as their interactions with low molecular weight regulatory agents, such as retinoids and steroids. The Laboratory is involved with the total spectrum of studies that can be done with growth factors, ranging all the way from mutating their chemical structure with the most advanced techniques of recombinant DNA research to evaluating their potential therapeutic usefulness as clinical agents in patients with defective wound healing.

We have characterized and purified to homogeneity a new peptide growth factor called transforming growth factor-beta (TGF-beta). Although this peptide was named for its ability to cooperate with members of the epidermal growth factor family to induce phenotypic transformation and anchorage-independent growth of non-neoplastic fibroblasts, we have recently shown that TGF-beta can inhibit the anchorage-independent growth of certain tumor cells. This bifunctional character of TGF-beta is best shown in experiments using fibroblasts transfected with the *myc* gene; in these cells TGF-beta can either stimulate or inhibit the anchorage-independent growth of the cells depending on the complete set of other growth factors operant on the cells.

Human platelets are a major storage site for type beta transforming growth factor; they contain 100 to 1000-fold more of this peptide than do other cells which have been examined to date. TGF-beta can be purified from platelets in a two-step procedure that involves sequential gel filtration in the absence and then presence of denaturant. Structural studies on homogeneous TGF-beta show that it is composed of two 12,500 dalton subunits which are held together by disulfide bonds. Platelets also contain smaller amounts of an EGF-like

peptide which can synergize with platelet TGF-beta to induce growth of NRK fibroblasts in soft agar. Mechanistically, these two peptides interact; incubation of purified TGF-beta with NRK cells specifically increases the number of receptors for epidermal growth factor.

The complete amino acid sequences for human TGF-alpha and TGF-beta have been deduced from the cDNA nucleotide sequence for each of these peptides cloned in collaboration with Genentech, Inc. Each of these peptides is synthesized as a part of a larger precursor molecule, and the messenger RNAs encoding each of these peptides are considerably larger than expected. TGF-alpha, a single-chain peptide of 50 amino acids, has been expressed in milligram quantities in *E. coli*; the recombinant peptide is fully active biologically. TGF-beta is a homodimer with each chain composed of 112 amino acids containing 9 cysteine residues; it has not yet been expressed in a biologically active form.

The human cDNA clones for both TGF-alpha and TGF-beta have been used for Northern blot analysis of total cytoplasmic RNA from several human tumor cell lines. The human TGF-beta cDNA clone can be used to detect TGF-beta mRNA in retrovirus-transformed rodent cell lines. The human, rat, and mouse TGF-beta mRNAs are of similar molecular weight (2.4 kb) on denaturing gels. Cloning of the rat TGF-beta gene will both permit direct comparison with the human gene and provide a high stringency probe to investigate mechanisms of transformation of rodent cell lines.

We have identified a single high affinity binding site for TGF-beta on NRK fibroblasts, indicating that both the positive and negative growth regulatory effects of TGF-beta on this cell must be mediated through the same receptor. Every cell type assayed to date possesses a similar high affinity receptor for TGF-beta, which appears to be constitutively expressed under all conditions, since none of the agents that modulate TGF-beta action appear to affect the receptor, and TGF-beta itself causes only a partial down-regulation of the receptor. The receptor appears to be a high molecular weight monomeric protein which, unlike other growth factor receptors, does not undergo ligand-induced clustering or phosphorylation.

There is now a solid base of data supporting a bifunctional role for TGF-beta in the regulation of cellular proliferation, cellular differentiation, and cell function. For example, TGF-beta synergizes with EGF and with PDGF to stimulate anchorage-independent growth of certain cells, while it blocks the mitogenic effects of EGF and PDGF on growth of the same cells in monolayer culture. TGF-beta also has profound effects on lymphocytes and can block the mitogenic effects of interleukin-2 on T-cells and can also block secretion of immunoglobulins by B-cells.

TGF-beta has recently been found to stimulate formation of extracellular matrix by cells of mesenchymal origin. Cells of the immune system and osteoblasts have been found to secrete relatively large amounts of TGF-beta, suggesting that fibrosis that accompanies chronic inflammation, as well as matrix formation by bone-forming cells may be dependent on TGF-beta. Investigations of embryonic development have shown unusually high expression of TGF-beta mRNA throughout embryogenesis; this, too, may be correlated with a requirement for matrix synthesis.

We have synthesized the complete rat TGF-alpha gene and expressed it in a retrovirus vector. NRK cells have been infected with recombinant viruses carrying this TGF-alpha gene, and these cells have the transformed phenotype in the presence of TGF-beta.

Studies have been performed on the levels of TGF-beta in monocytes, both before and after their activation to macrophages. Activation causes a definite increase in the production of TGF-beta mRNA, as well as an increase in secreted protein. These studies are of major importance with respect to the role of the macrophage as a key mediator in inflammatory reactions.

Polyclonal antibodies have been raised against human TGF-beta. Immunoglobulin fractions have been prepared from this serum by affinity chromatography. These antibodies effectively block binding of TGF-beta on NRK cells. In collaboration with investigators in the Laboratory of Human Carcinogenesis, we have used this antiserum to demonstrate that TGF-beta is the principal component in serum that induces the terminal differentiation of human bronchial epithelial cells.

In summary, the Laboratory of Chemoprevention is currently involved in some very new approaches to the control of the growth of cancer cells. These approaches have led to the discovery of new growth factors which themselves may be useful therapeutic agents, as well as to current attempts to synthesize "fraudulent" growth factors or to utilize other mechanisms to antagonize the effects of undesirable growth factors in the cancer cell. Considering the progress that has been made in the past 5 years, one may be cautiously optimistic that this area of investigation will continue to provide significant results.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05051-08 LC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Mechanism of Action of Type Beta Transforming Growth Factor		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Anita B. Roberts	Staff Scientist LC NCI
Others:	Sonia B. Jakowlew	Senior Staff Fellow LC NCI
	Paturu Kondaiah	Visiting Fellow LC NCI
	Joseph M. Smith	Biologist LC NCI
	Nannette B. Roche	Biologist LC NCI
	Pamela J. Dillard	Chemist LC NCI
COOPERATING UNITS (if any) J. H. Kehrl and A. S. Fauci, Laboratory of Immunoregulation, NIAID		
LAB/BRANCH Laboratory of Chemoprevention		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	3.0	2.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The purpose of the project is to determine the mechanism of action of type beta transforming growth factor (TGF) both in cell culture in vitro and in animal studies in vivo and to determine the relatedness of peptides functionally equivalent to TGF-beta in different species. Mechanistically, particular emphasis will be placed on identification of the switching mechanisms active in control of the bifunctional responses of cells to TGF-beta. Thus far, three different classes of molecules have been found to influence the response of cells to TGF-beta; these include 1) other polypeptide growth factors such as TGF-alpha, epidermal growth factor, and platelet-derived growth factor; 2) retinoids and other low-molecular weight effectors; and 3) oncogenes and their polypeptide products. Each of these classes of substances is known to affect gene expression. Tools that will be used to assess TGF-beta function in this project will include specific bioassays, receptor assays, immunoassays, and assays for TGF-beta messenger RNA. Mechanistic studies are aimed at two levels: in the whole animal and in both primary cells and established cell lines in culture. To determine the relatedness of TGF-betas from different species, classical gene-cloning methodology will be used to identify and sequence complementary DNA sequences coding for TGF-beta in both the pig and the chicken.</p>		

POSITION DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Anita B. Roberts	Staff Scientist	LC	NCI
Sonia B. Jakowlew	Senior Staff Fellow	LC	NCI
Paturu Kondaiah	Visiting Fellow	LC	NCI
Joseph M. Smith	Biologist	LC	NCI
Nannette B. Roche	Biologist	LC	NCI
Pamela J. Dillard	Chemist	LC	NCI

Objectives:

This project is directed towards understanding the relatedness of TGF-betas in different species and the mechanisms controlling the bifunctional effects of TGF-beta on cells. It includes a study of mechanistic overlaps between the actions of growth factors, oncogenes or oncogene products, and small effector molecules such as the retinoid on cells. An effort will be made to clarify the mechanistic basis for the bifunctional effects of both retinoids and TGFs on proliferation and transformation as well as for the observations that interactions between retinoids and TGFs are antagonistic in certain situations and synergistic in others. One specialized aspect of TGF-beta is its ability to induce a local fibrotic response in vivo. Efforts will be focused on identifying the specific cell types involved in this response and studying their responses to TGF-beta in in vitro culture.

Methods Employed:

Standard methods are utilized based on use of specific reagents such as iodinated TGF-beta, antibodies to TGF-beta, and cDNA probes for growth factors, oncogenes, and matrix proteins.

Major Findings:

There is now a solid base of data supporting a bifunctional role for TGF-beta in the regulation of cellular proliferation, cellular differentiation, and cell function. For example, TGF-beta synergizes with EGF and with PDGF to stimulate anchorage-independent growth of certain cells, while it blocks the mitogenic effects of EGF and PDGF on growth of the same cells in monolayer culture. TGF-beta also has profound effects on lymphocytes and can both block the mitogenic effects of interleukin-2 on T-cells and block secretion of antibodies by B-cells. The mechanisms of these effects are not yet understood.

TGF-beta stimulates matrix production by mesenchymal cells. Cells of the immune system and osteoblasts have each been shown to secrete TGF-beta, suggesting that both fibrosis that accompanies chronic inflammation and matrix formation by bone-forming cells may be dependent on TGF-beta. This mechanism is probably also operative in tissue repair where TGF-beta is released from platelets, macrophages, and lymphocytes to stimulate matrix synthesis by target mesenchymal cells. Investigations into embryonic development show unusually high expression of

TGF-beta mRNA throughout the pre-natal period, again possibly correlating with the need for matrix synthesis. Whether TGF-beta acts on target cells to increase mRNA for matrix proteins is currently under investigation.

Two forms of TGF-beta are found in bovine bone. We are currently investigating whether this finding is universal and whether the two forms utilize the same or different cellular receptors.

Publications:

Anzano, M. A., Roberts, A. B., De Larco, J. E., Wakefield, L. M., Assoian, R. K., Roche, N. S., Smith, J. M., Lazarus, J. E., and Sporn, M. B.: Increased secretion of type beta transforming growth factor accompanies viral transformation of cells. J. Molec. Cell. Biol. 5: 242-247, 1985.

Anzano, M. A., Roberts, A. B., and Sporn, M. B.: Anchorage-independent growth of primary rat embryo cells is induced by platelet-derived growth factor and inhibited by type-beta transforming growth factor. J. Cell. Physiol. 126: 312-318, 1986.

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Kehrl, J. H., Wakefield, L. M., Roberts, A. B., Jakowlew, S., Alvarez-Mon, M., Derynck, R., Sporn, M. B., and Fauci, A. S.: The production of TGF-beta by human T-lymphocytes and its potential role in the regulation of T-cell growth. J. Exp. Med. 163: 1037-1050, 1986.

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Rook, A. H., Kehrl, J. H., Wakefield, L. M., Roberts, A. B., Burlington, D. B., Lane, H. C., Sporn, M. B., and Fauci, A. S.: Effects of transforming growth factor-beta in the functions of natural killer cells: Depressed cytolytic activity and blunting of interferon responsiveness. J. Immunol. 136: 3916-3920, 1986.

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Stern, D. F., Roberts, A. B., Roche, N. S., Sporn, M. B., and Weinberg, R. A.: Differential responsiveness of myc- and ras-transfected cells to growth factors: Selective stimulation of myc-transfected cells by EGF. Mol. Cell. Biol. 6: 870-877, 1986.

Tashjian, Jr., A. H. Voelkel, E. F., Lazzaro, M., Singer, F. R., Roberts, A. B., Derynck, R., Winkler, M. E., and Levine, L.: Alpha and beta human transforming growth factors stimulate prostaglandin production and bone resorption in cultured mouse calvaria. Proc. Natl. Acad. Sci. USA 82: 4535-4538, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05267-05 LC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Identification and Action of Platelet-derived Transforming Growth Factor-beta		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Richard K. Assoian	Staff Fellow LC NCI
Others:	Barbara Fleurdelys	Bio. Lab. Tech. LC NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Chemoprevention		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	2.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Human platelets were extracted with acid-ethanol and platelet-derived TGF-beta was purified from the extract by a two-column procedure using sequential gel filtration in the absence and then presence of urea. Purified TGF-beta is a protein of 25,000 daltons, and it is comprised of two 12,500 dalton subunits held together by disulfide bonds. The purified factor elicits its biological activity at concentrations less than 4pM. Comparative studies showed that platelets contain 100 times more TGF-beta than do other non-neoplastic tissues. Platelets also contain a peptide growth factor related to EGF. These two new growth factors can interact mechanistically. Incubation of TGF-beta with NRK cells for 6 h results in an increased number of cell surface EGF receptors. IGF-II receptors are not affected. Shorter incubations with TGF-beta show that this peptide can also increase the Kd of the high affinity EGF receptor. TGF-beta has bifunctional effects on explant cultures of bovine vascular smooth muscle cells; it inhibits monolayer growth yet promotes growth in soft agar. TGF-beta is also produced by activated monocytes.		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Richard K. Assoian	Staff Fellow	LC	NCI
Barbara Fleurdelys	Bio. Lab. Tech.	LC	NCI

Objectives:

To examine the role of bioactive peptides in modulating normal and neoplastic cell growth. Emphasis will be placed on 1) the isolation of transforming growth factors (TGFs) from platelets and 2) the mechanism by which platelet-derived TGF elicits a transformed phenotype and controls growth of mesenchymal cells.

Methods Employed:

Clinically outdated human platelets are extracted with acid-ethanol and the soluble peptides are precipitated with ether. The extract is purified by gel filtration, high pressure liquid chromatography and preparative gel electrophoresis. Biological activity is localized by use of an anchorage-independent growth assay with NRK fibroblasts. Peptides are chemically localized by polyacrylamide gel electrophoresis in conjunction with silver staining and analytical radioiodination. Purified TGF-beta is incubated with cultures of primary and established cell lines. EGF receptors are measured with a radioreceptor assay. EGF action is measured on NRK cells by synergism with TGF-beta in soft agar or stimulation of ³H-thymidine incorporation in monolayer. TGF-beta is measured by soft agar and receptor competition assays.

Major Findings:

Platelets are the major non-neoplastic source of TGF-beta. The platelet-derived factor has been purified to homogeneity and shown to be a protein of 25,000 daltons comprised of two 12,500 dalton subunits. Disulfide bonds are involved in holding the subunits together. The primary structure of TGF-beta has been determined. Platelets also contain a peptide ($M_r=27,000$) which competes with labeled EGF for EGF receptor binding. TGF-beta in monolayer culture has a biphasic effect on EGF binding. It induces a transient decrease in receptor binding (due to a decrease in affinity) followed by a prolonged stimulation of binding (due to an increase in receptor number). Both of these effects have direct functional consequences for EGF-stimulated mitosis. TGF-beta also affects explant cultures of bovine aortic smooth muscle cells by inhibiting monolayer growth and stimulating soft agar growth. The data identify a primary cell that behaves similarly to NRK fibroblasts with regard to TGF-beta action. TGF-beta mRNA and protein is produced by activated monocytes.

Publications:

Assoian, R. K.: Biphasic effects of type beta transforming growth factor on epidermal growth factor receptors in NRK fibroblasts: Functional consequences for epidermal growth factor-stimulated mitosis. J. Biol. Chem. 260: 9613-9617, 1985.

Assoian, R. K., Grotendorst, G. R., Miller, D. M. and Sporn, M. B.: Three peptide growth factors from human platelets coordinating phenotypic transformation. Nature 309: 804-806, 1984.

Assoian, R. K. and Sporn, M. B.: Type beta transforming growth factor in human platelets: Release during platelet degranulation and action on vascular smooth muscle cells. J. Cell Biol. 102: 1217-1223, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05396-03 LC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Development of Analogs for Study of Oncogenesis and Development of the Rat		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Shinichi Watanabe	Senior Staff Fellow LC NCI
Other:	Eliane M. Lazar	Visiting Fellow LC NCI
	Myung Kim	Visiting Fellow LC NCI
	Kondaiah Paturu	Visiting Fellow LC NCI
	Ellen Van Obberghen	Staff Fellow LC NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Chemoprevention		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 3.2	PROFESSIONAL: 3.2	OTHER: 0.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Several mutations have been introduced into a cloned human TGF-alpha gene by site-directed mutagenesis. These mutant forms of TGF-alpha were expressed in yeast expression vectors. Some of them show altered characteristics when compared to normal (wild type) TGF-alpha. The rat TGF-alpha gene was chemically synthesized and expressed in a retrovirus vector. Infectious recombinant retrovirus carrying rat TGF-alpha makes NRK cells grow in soft agar in the presence of TGF-beta. Infected NRK cells secrete rat TGF-alpha at a higher level than most transformed cells. The rat TGF-alpha has been inserted in <u>E. coli</u> plasmid which has a strong promoter. The rat TGF-alpha gene has also been fused with HBsAg gene to express as fusion protein in eukaryote expression vector.		

PROJECT DESCRIPTIONNames, Title, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Shinichi Watanabe	Senior Staff Fellow	LC	NCI
Eliane M. Lazar	Visiting Fellow	LC	NCI
Myung Kim	Visiting Fellow	LC	NCI
Paturu Kondaiah	Visiting Fellow	LC	NCI
Ellen Van Obberghen	Staff Fellow	LC	NCI

Objectives:

We are studying the role of TGF- α in normal and transformed cells and the structure-function relationship of TGF- α .

Methods Employed:

Chemically synthesized oligonucleotides were used to synthesize mutagenized genes. All genes were expressed in bacterial, yeast, and animal virus expression vectors. Infectious recombinant virus was used to infect cells in vitro to show production of functional TGF- α . TGF- α expressed from other systems were tested on indicator cells.

Major Findings:

We have synthesized the complete rat TGF- α gene and expressed it in a retrovirus vector. Only the TGF- α gene with a leader sequence in the retrovirus vector produced TGF- α at higher levels than transformed cells. Although synthetic rat TGF- α gene does not contain 3' processed region, mature TGF- α can be folded correctly and secreted. Several mutations in human TGF- α showed that any disruptions of disulfide bonds abolish activity of TGF- α . Leu at amino acid position 47 can be substituted to Ala, but Asp at amino acid position 48 cannot be substituted for Ala.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05398-03 LC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Functional Characterization of Transforming Growth Factor Beta and its Receptor		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: Michael B. Sporn	Chief	LC NCI
Other: Lalage Wakefield	Visiting Fellow	LC NCI
Bradford O. Fanger	Guest Researcher	LC NCI
Diane M. Smith	Biologist	LC NCI
Tohru Masui	Visiting Fellow	LHC NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Chemoprevention		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: <div style="text-align: center;">2.8</div>	PROFESSIONAL: <div style="text-align: center;">2.0</div>	OTHER: <div style="text-align: center;">0.8</div>
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> Transforming growth factors (TGFs) are acid-stable polypeptides that induce reversible phenotypic transformation of normal indicator cells. The purpose of this project is to determine the role that endogenously-produced TGF-beta may play in the growth of normal and transformed cells and to characterize its mode of action at a biochemical level. To this end, polyclonal antisera have been raised against TGF-beta and development of monoclonal antibodies is in progress. The effects of these antibodies on the anchorage-dependent and -independent growth of normal and transformed cells are being investigated and will be analyzed in terms of the biochemical functions affected. Since the first step in the interaction of TGF-beta with the cell is binding of the growth factor to the cell surface, initial investigations have concentrated on the characterization of cell surface receptors for TGF-beta. Development of a radio-receptor assay for TGF-beta has allowed identification of a specific high affinity receptor for TGF-beta on all normal and transformed cell lines studied so far. The receptor appears to be a disulphide-linked dimer that does not undergo ligand-induced autophosphorylation or clustering. Receptor properties are modulated by transformation but binding of TGF-beta to its receptor does not appear to be a major control point in TGF-beta action. Normal and transformed cells have been shown to secrete TGF-beta in an inactive form. Certain tumor cells have lost the ability to activate this latent form and thus cells can no longer limit their own growth in an autocrine manner. The nature of the latent form and of the activation process are being investigated, since activation is potentially an important control point in TGF-beta action and may become disrupted in transformed cells. Further characterization of the role of endogenously produced TGFs and their interaction with the cell surface receptor should help elucidate the role these molecules play in the process of carcinogenesis. </p>		

PROJECT DESCRIPTIONNames, Title, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project

Lalage M. Wakefield	Visiting Fellow	LC	NCI
Bradford O. Fanger	Guest Researcher	LC	NCI
Diane M. Smith	Biologist	LC	NCI

Objectives:

The purpose of this project is to examine the role of transforming growth factor beta (TGF) in the control of normal cell growth and in the process of malignant transformation. Anti-TGF-beta antibodies are being employed to investigate the involvement of endogenously-produced TGF-beta in growth control in normal and transformed cells. The mechanism of action of this growth factor is being studied at a biochemical level with particular emphasis on the activation of the latent form of TGF-beta secreted by cells in culture and on the initial interaction of the TGF-beta with the cell surface receptor. With a greater understanding of mechanism of action of TGF-beta in transformation, analogs and potential inhibitors may be synthesized and tested with a view to producing effective chemotherapeutic agents.

Methods Employed:

A method has been developed for the radioiodination of TGF-beta to high specific activity for use in specific radioreceptor assays to characterize TGF-beta binding to a variety of cell lines in culture. Binding data are subjected to Scatchard analysis. Biochemical characterization of the receptor has involved chemical cross-linking of radiolabeled ligand to the receptor followed by analysis of cross-linked species by SDS polyacrylamide gel electrophoresis and autoradiography. TGF-beta activity secreted by cells is quantitated using competitive radioreceptor assays and is characterized biochemically by immunoprecipitation and Western blot techniques using specific anti-peptide antisera. The latent form of TGF-beta is being purified by fast protein liquid chromatography. The ability of cells to grow in an anchorage-independent manner in soft agar is used as an assay for the transformed phenotype and the number and size of cell colonies obtained is measured using an Omnicon image analyzer. Anchorage-dependent growth is quantitated by determining changes in cell number for cells grown in monolayer.

Major Findings:Characterization of the TGF-beta Receptor

Binding of TGFs to the cell membrane initiates the chain of events leading to cell transformation, so TGF-receptor interaction and its modulation are of particular interest in any mechanistic studies. A method was developed for the iodination of TGF-beta without loss of biological activity for use in a radioreceptor assay. High affinity TGF-beta receptors have been found on all of over 60 cell types assayed to date, including cells of mesenchymal, epithelial and hematopoietic origin and many tumor-derived cells. There is a strong

inverse relationship between the affinity of the receptor for TGF-beta and the number of receptors expressed per cell indicating a striking degree of conservation of the level of ligand binding. None of the agents that modulate TGF-beta action affect the binding of TGF-beta to target cells suggesting modulation of TGF-beta binding is not a major control point in TGF-beta action. Unlike the PDGF or EGF receptors, the receptor for TGF-beta does not show extensive ligand-induced down-regulation and in some cell types down-regulation does not occur at all. In addition, there is never more than a two-fold change in TGF-beta receptor numbers on cell transformation, again in contrast to observations with other growth factor receptors. Thus, the TGF-beta receptor appears to be unique in the degree to which the level of TGF-beta binding is conserved in different cell types under different conditions, probably reflecting a very critical role for TGF-beta in the regulation of growth and differentiation in many cell types. Chemical cross-linking studies have shown the TGF-beta receptor to be a disulphide-linked dimer with a molecular weight of approximately 560 Kd. Unlike most other growth factor receptors, the TGF-beta receptor does not appear to undergo ligand-induced clustering or phosphorylation. A soluble receptor binding assay has been developed which will aid in the purification and further characterization of the receptor and associated enzyme activities.

Paracrine and Autocrine Growth Control by Endogenous TGF-beta

Polyclonal antibodies have been raised against human platelet TGF-beta. Immunoglobulin fractions have been prepared from this serum by affinity chromatography on protein A-sepharose. These antibodies effectively block binding of iodinated TGF-beta to its receptor and inhibit the transforming effect of exogenously-added TGF-beta on NRK cells. In a collaboration with Tohru Masui (NCI:LHC), this anti-serum was used to demonstrate that TGF-beta is the major component in serum that induces the terminal differentiation of normal human bronchial epithelial cells. These cells spontaneously cease to divide and undergo squamous differentiation at high density in serum-free culture. The conditioned medium from high density cultures contains endogenously-secreted TGF-beta at a concentration above the threshold required to inhibit growth and induce differentiation. Since the cells have functional receptors for TGF-beta, the results indicate that the growth of normal human bronchial epithelial cells may be regulated by endogenous TGF-beta in a negative autocrine manner. Various human lung carcinoma lines are being studied to determine whether the tumorigenic lesion in any case is a disruption of the TGF-beta autocrine loop.

Identification and Characterization of Latent TGF-beta

Using a competitive radioreceptor assay to quantitate TGF-beta, it was shown that all of 12 normal and transformed cell types assayed to date secrete TGF-beta in a latent form, activatable by transient acidification. The human lung carcinoma line A549 appears to have lost the ability to activate the latent endogenous form of TGF-beta and can therefore no longer limit its own growth in an autocrine fashion, whereas the parent normal cell can respond to the latent TGF-beta that it secretes. Thus, activation of latent-secreted TGF-beta may be a step that is disrupted on transformation. Gel filtration data indicates two high molecular weight forms of latent TGF-beta with M_r of 700,000 daltons and 100,000 daltons. The biochemical nature of these forms is being probed using antisera to mature platelet TGF-beta and precursor peptide sequences.

Publications:

Fanger, B. O., Wakefield, L. M., and Sporn, M. B.: Structure and properties of the cellular receptor for transforming growth factor type beta. Biochemistry 25: 3083-3091, 1986.

Masui, T., Wakefield, L.M., Lechner, J. F., Laveck, M. A., Sporn, M. B., Harris, C. C.: Type beta transforming growth factor is the primary differentiation-inducing serum for normal human bronchial cells. Proc. Natl. Acad. Sci. USA 83: 2438-2442, 1986.

ANNUAL REPORT OF
THE LABORATORY OF COMPARATIVE CARCINOGENESIS
CHEMICAL AND PHYSICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1985 through September 30, 1986

The Laboratory of Comparative Carcinogenesis (LCC) plans, develops and implements a research program in experimental carcinogenesis. The Laboratory (1) compares effects of chemical carcinogens in rodents and nonhuman primates to identify determinants of susceptibility and of resistance to carcinogenesis; (2) identifies, describes, and investigates mechanisms of interspecies differences and of cell and organ specificities in carcinogenesis; (3) investigates the roles of nutrition, metabolism, the perinatal age period and pregnancy in modifying susceptibility to chemical carcinogens; and (4) conducts biological and morphologic studies on the pathogenesis of naturally occurring and induced tumors in experimental animals.

Summary Report: The Laboratory of Comparative Carcinogenesis provides a major focus within the Chemical and Physical Carcinogenesis Program for studies on the mechanisms of chemical carcinogenesis that involve primary neoplasia in animals as experimental endpoints. An increasing volume of evidence continues to support the hypothesis that for many, if not most tissues, transient exposure to chemical carcinogens, either before or after birth, may be necessary but is not sufficient to elicit tumor development. The widely differing patterns of organ specificity that frequently occur in experimental carcinogenesis in different species, even in studies with direct-acting agents that are independent of cellular metabolism, are, in many cases, not explicable on the basis of toxicodynamics nor on the basis of differential capacity to repair damage in different tissues. These findings complicate efforts at human risk assessment based on the extent of reaction or persistence of binding products of carcinogens in known animal and putative human target tissues. In addition, more and more agents are being identified that cause tumors in experimental rodents but do not react chemically with cellular constituents including DNA, i.e., are not genotoxic. The fact that potential tumor cells may remain latent for large fractions of a lifetime in experimental animals and that increasing numbers of nongenotoxic agents are being discovered which promote proliferation of such latent cells to form preneoplastic lesions that progress to neoplasia suggest that the phenomenon of tumor promotion may be of great significance for the genesis of human cancer.

There is, at present, no unifying hypothesis for the general mechanism of action of tumor promoters other than the concept that such agents reduce intercellular communication. Furthermore, most experimental studies on tumor promotion in specific tissues or organ systems have focused on one, or at most, two species, and the empirical data base from which mechanistic hypotheses of tumor promotion will eventually emerge remains very narrow. Accordingly, there is no certainty, at present, that agents identified as promoters in rodent tissues will have similar effects in other species, including man. In order to expand the limited data base on organ specificity and interspecies correlations in tumor promotion, a major coordinated program has been established in this Laboratory to identify previously unsuspected promoting agents; to establish rigorously the limits of cellular specificity for tumor promotion by specific agents; to compare dose/effect relationships from one species to another, including both rodent and nonhuman primate species; and to utilize these data in mechanistic investigations on the phenomena of tumor promotion. Identification of

specific transforming genes (oncogenes) in both human and experimental tumors has stimulated great interest and intensive efforts in many laboratories to clarify the roles of those genes in the pathogenesis of cancer. As many of the known oncogenes derive from normal elements of the mammalian genome (Hunter, T., JNCI 73: 773-785, 1984), there is a real possibility that the biochemical mechanisms of neoplastic transformation may be definable by thorough analysis of the properties of the oncogenes and their cellular homologs. Of special interest in chemical carcinogenesis are oncogenes that behave as dominant genetic elements and that are activated to this behavior either by single-base transition mutation, such as the ras family of genes (Sukumar, S. et al., in Genes and Cancer, Liss, New York, 1984, pp. 353-371) or by chromosomal rearrangement, as in the case of myc (Alitalo, K., et al., PNAS 80: 1701-1711, 1983). Both of these mechanisms of activation can be caused in principle by genotoxic chemical carcinogens, which can be provisionally identified as such by their mutagenic or clastogenic effects. The mechanisms of action of such agents, long considered to involve damage to DNA, may eventually be reconciled with molecular virology, and the crucial events in cellular transformation by both chemical and biological agents defined, through analysis of the activation and behavior of oncogenes. Detection and critical evaluation of the roles of dominant oncogenes, especially mutant genes of the ras family, is also a major unifying theme in the research activities of several Sections within this Laboratory.

Major research programs on metabolic determinants of transplacental carcinogenesis in rodents and nonhuman primates, on carcinogenesis by salts of heavy metals, on the chemistry and biochemistry of nitrosamines, and on the role of nutritional deficiencies in chemical carcinogenesis also continue and are described in detail both in the following summary reports of each Section and in the individual project reports.

In response to recommendations made during peer review of the research of this Laboratory at its site visit in July 1985, a number of organizational changes and programmatic modifications are being made. New research projects also have been undertaken in response to the suggestion that efforts should be intensified on carcinogenic effects of substances known to be relevant to humans. New research projects on transplacental carcinogenesis by tobacco-specific nitrosamines and systematic studies on carcinogenesis and in vivo mutagenesis by fecapentaenes have been begun.

The Office of the Chief (1) organizes comparative research on mechanisms of chemical carcinogenesis in susceptible and resistant species of experimental animals, (2) arranges and fosters collaborative approaches to specific research projects involving several Sections within, and independent investigators outside, the Laboratory, and (3) provides general support and direction to the intramural research program of the Laboratory.

In the Developmental Biology Working Group, significant advances have been made in studying the role of activation of specific oncogenes in the course of chemical carcinogenesis in certain rodent target tissues. Extension of last year's report on the selective activation of K-ras oncogenes in the renal mesenchymal tumor of rats exposed once neonatally to methyl(methoxymethyl) nitrosamine have been extended and confirmed, and contrast markedly with demonstration of the selective activation of an oncogene, neu (erbB-2), in a high proportion of primary schwannomas of the peripheral, cranial, and spinal nerves induced by transplacental exposure to the direct acting alkylating agent N-nitrosoethylurea. This is in sharp contrast to the apparent absence of this gene in primary chemically induced tumors of the central nervous system and to the sporadic and irregular detection of genes of the ras family in primary chemically induced tumors of the rat liver and the rat intestine. In the systems which yield activated oncogenes in tumor tissues, the activated genes are

never found in DNA preparations from grossly normal tissues of the same animal including liver, brain, or kidney. Also, in all cases, the presence of rat repetitive sequences in the transformed NIH 3T3 target cells has been consistent with the postulated role of rat genes in the transformation of the indicator cells. Consistent demonstration of specific dominant transforming genes in different tumor types, and their absence from others, argues against the random and artifactual activation of these transforming genetic sequences in the process of DNA isolation or in the transfection procedure and provides additional evidence in favor of the interpretation that they play a significant role in the process of carcinogenesis in those tissues in which they are consistently found. The successful application, in collaboration with the Tumor Pathology and Pathogenesis Section, of the avidin-biotin peroxidase complex immunocytochemical technique to the localization and visualization of oncogene protein products in fixed tissue sections has been successfully achieved. Oncogene proteins have been found in Harvey virus-induced sarcomas, and progress is being made towards the demonstration of these proteins in chemically induced tumors in early stages of development, which is expected to yield important information on the role of activated oncogenes in different stages of the development of chemically induced neoplasms.

The Inorganic Carcinogens Working Group has demonstrated a new category of metal binding proteins in rodent testicular tissue that are not metallothionein, and whose capacity to bind carcinogenic metal cadmium clearly is insufficient to prevent carcinogenesis in that organ by the carcinogenic metal. Recognition that this protein, previously reported to be metallothionein, is different removes a major discrepancy in the evolving picture of the role of metallothioneins in protection of specific tissues from carcinogenesis by the carcinogenic metals.

The Perinatal Carcinogenesis Section investigates the induction of cancer in experimental animals before birth and during infancy; evaluates perinatal exposures to chemical carcinogens, inducers of xenobiotic metabolism, and tumor promoters as causative factors in pediatric and adult forms of human cancer; studies the effects of exposure to carcinogens during pregnancy; and investigates the relation of cellular differentiation to perinatal susceptibility to chemical carcinogens and to the consequent development of neoplasia.

A major goal of the Perinatal Carcinogenesis Section is the identification and mechanistic study of the factors that affect and modulate the causation and appearance of tumors originating from transformed cells in the fetus or neonate. These factors may include maternal processing of carcinogens, interactions of these chemicals with the immature organs, and influences on the development of the initiated neoplasms. The demonstration that certain oncogenes, especially those of the ras and neu families, can be activated to dominant transforming genes by simple transition mutations and that these genes are specifically and characteristically found in tumor tissue, but not normal tissues, in carcinogen-treated animals has prompted the study of transplacental mutagenesis as a probe for organ-specific mutagenic and presumably carcinogenic genotoxicity. Susceptibility of the rodent fetus to transplacental mutagenic effects of N-nitrosoethylurea was found to vary greatly during prenatal development and to be characterized by a pronounced maximum at day 9 of gestation in the Syrian hamster and at day 11 in the rat; in both cases, maximum susceptibility to induction of mutations occurred toward the end of organogenesis and before or approximately concurrent with the earliest beginnings of susceptibility to tumor induction measured by the actual occurrence of tumors in transplacentally exposed offspring during postnatal life. This is consistent with the concept that a large proportion of potentially transformed cells remain phenotypically normal throughout life unless there is a subsequent encounter with an effective tumor promoting agent.

In pharmacogenetic investigations of transplacental carcinogenesis by polynuclear aromatic hydrocarbons in mice, genetic backcrosses of selected inbred strains were employed to produce fetuses that were, in the same mother, either inducible or noninducible for enzymes that metabolize this class of carcinogenic agents. When hydrocarbon treatment was preceded by exposure of the mother to noncarcinogenic enzyme inducers (beta-naphthoflavone) the numbers of tumors in both lung and liver were reduced, but only in induction-responsive fetuses. Both maternal and fetal genotypes, with respect to induction of hydrocarbon metabolism, impacted significantly on the consequences to the fetus of prenatal exposure to aromatic hydrocarbon carcinogens.

The Tumor Pathology and Pathogenesis Section (TPPS) (1) characterizes the biology and pathology of naturally occurring and experimentally induced preneoplastic and neoplastic lesions of laboratory animals; (2) uses morphologic, histochemical and ultrastructural methods to define the pathogenesis of experimental tumors; (3) develops animal models to aid in understanding causes, pathogenesis and pathology of human cancers; and (4) provides guidance, consultation and collaboration in tumor and laboratory animal pathology to investigators and scientists in other Sections of the Laboratory.

The pathology and biology of experimentally induced and naturally occurring neoplasms of rodents are characterized and compared in order to elucidate the mechanisms of induction and progression. Pathology and histogenesis of individual tumor types are investigated with the use of serial sacrifice studies, immunocytochemistry, automated image analysis with stereology, conventional light microscopy, ultrastructure and histochemistry. Computerized image analysis of early and late induced focal proliferative lesions was used, together with stereologic techniques, to demonstrate that small focal hyperplastic lesions progressively grew in size to develop into adenomas and carcinomas. Detailed histogenesis investigations were performed for mouse, rat and hamster liver, hamster upper respiratory tract, and mouse lung.

Retroviral proteins and viruses have been localized to cells in fixed tissue sections of monkeys and humans with acquired immune deficiency syndrome caused by lentiviruses or type D retroviruses. The viral antigens have been seen in abundance during early stages of the diseases in both species while low levels are observed as the diseases progress. Unlike findings in virally-induced leukemias of rodents and cats, these viruses are not found in megakaryocytes or myeloid cells but are commonly seen within glia and cerebral macrophages. This technique will aid in the understanding of the pathogenesis of these diseases. Most recently, these viruses have been found to infect our experimental monkeys and our new application of the ABC technique should help us characterize the findings in our monkeys.

Tumor promotion in nonsquamous epithelia in rats and mice has been shown to be effected in a markedly specific pattern by long-acting sedative barbiturates and to involve the widest variety of unrelated epithelial tissue types known to be affected by tumor promoting agents. Promotion was demonstrable in thyroid follicular epithelium, liver parenchyma, renal cortical tubular epithelium, and urothelium, but was consistently demonstrated only in thyroid and liver, with effects in other tissues markedly dependent on the chemical structure of the barbiturate promoter. Short-acting hypnotic barbiturates, the kinds most commonly used in anesthesia in humans, have not been found to promote carcinogenesis; this good news, however, must be tempered by the demonstration that the benzodiazepine tranquilizers, diazepam and oxazepam, are as potent promoters for the mouse (but not the rat) liver parenchyma as is the barbiturate phenobarbital. Susceptibility to promotion has been shown to be a genetically determined trait that is inherited in a Mendelian dominant manner; and genetic analysis is in progress to quantify, locate, and ultimately to identify,

clone, and sequence the genes that confer susceptibility to tumor promotion in specific target organs. A close correlation has been established between the capacity of agents to promote carcinogenesis in the liver and to induce biosynthesis of specific drug metabolizing enzymes of the P-450-dependent monooxygenase group, providing additional basis for the concept that tumor promoters act not simply by releasing a latent neoplastic cell from the constraints on growth conferred through intercellular communication, but through inductive processes on gene expression that serve also to alter the promoted cells from which tumors develop.

The Nutrition and Metabolism Section (1) investigates the effects of dietary constituents on target tissue susceptibility to chemical carcinogenesis and (2) studies mechanisms by which dietary constituents, such as methyl donors or their metabolites, alter carcinogenic processes.

The Section has focused its interest on the role of lipotropes, methionine, choline, vitamin B₁₂ and folic acid in chemical carcinogenesis. The chronic administration of diets devoid of methionine and/or choline has been shown to promote the formation of hepatocellular carcinomas in the livers of F344 rats and B6C3F1 mice initiated with DEN. Administration of diets devoid of both methionine and choline led to the formation of metastatic hepatocellular carcinomas in both species, even in the absence of any further treatment with hepatocarcinogens. Chronic feeding of diets lacking both methionine and choline to F344 rats caused a decrease in the 5-methyldeoxycytidine contents of hepatic DNA. Chronic administration of the hepatocarcinogen, ethionine, causes similar decreases in hepatic 5-methyldeoxycytidine. However, male C3H mice were insensitive to the hepatocarcinogenic and DNA hypomethylating activities of methionine- and choline-deficient diets. Further evidence that methyl insufficiency exerts a causative role in hepatocarcinogenesis in vivo is provided by the observation that tumor promotion and causation in the livers of C3H mice treated with phenobarbital are inhibited by high dietary levels of methionine.

Recent in vitro studies also provide evidence of a contributing role of methyl insufficiency in carcinogenesis. Deazaadenosine, an inhibitor of DNA methylation, transforms rat liver cells in culture. Finally, transfection of NIH 3T3 cells has been achieved with DNA isolated from a small fraction (3/25) of hepatocellular carcinomas produced in F344 rats initiated with diethylnitrosamine and subsequently fed the methyl-deficient diets. Such transfecting activity has been associated with a hypomethylated c-Ha-ras gene in the carcinomas.

These results provide interesting evidence that a physiological insufficiency of methyl donors, possibly acting via hypomethylated DNA, contributes significantly to hepatocarcinogenesis in certain rodents. Results from this and other laboratories have shown clear associations between methyl insufficiency, DNA hypomethylation and tumor formation, even in humans. Establishment of a causal relation between methyl insufficiency and tumor formation in a variety of tissues and cell types would be of major significance in understanding the etiology of cancer.

The Chemistry Section (1) plans and conducts laboratory research on the chemistry of organic and inorganic carcinogens; (2) investigates mechanisms of carcinogen formation, with the aim of understanding and ultimately preventing formation of such compounds; (3) studies chemical reactivity of carcinogens to identify reaction paths and products causally related to tumor formation as well as alternative pathways that may destroy carcinogens or otherwise interrupt carcinogenic reaction sequences; and (4) conducts comparative investigations of molecular interactions between chemical carcinogens and the cells of different organs and species to identify factors that contribute to organ specificity and species differences in chemical carcinogenesis.

Mechanisms potentially responsible for formation, activation, and detoxification of carcinogenic N-nitroso compounds in the human body are under intensive investigation. The bifunctional amine, piperazine, which is used as an anthelmintic in humans, has been shown to react with nitrite with remarkable facility under catalysis by certain iron complexes. The results suggest that similar catalysis by biological iron compounds may be responsible for some endogenous formation of nitrosamines in vivo. Activation studies have revealed that many volatile anesthetics act as competitive inhibitors of nitrosamine metabolism and that N-nitrosomethylethylamine can 2-hydroxyethylate as well as methylate and ethylate DNA. The latter result was predicted on the basis of deuterium isotope effect investigations. The deuterium isotope effect on conversion to carbon dioxide of formaldehyde and formate, one-carbon intermediates in the oxidation of N-nitrosodimethylamine (NDMA), has been found to be negligible. The extent of denitrosation, a potentially very important detoxification route for NDMA, has been estimated in vivo from urinary methylamine excretion data. Refinements of destruction methods for carcinogenic N-nitroso compounds have been introduced and several salts of nitrosamines with strong acids have been prepared.

The Ultrastructural Studies Section plans and conducts research to investigate the differentiation of potentially neoplastic epithelial cells and its relation to phenotypic expression of the neoplastic genotype with special emphasis on various cell interactions during the transformation process. Our special goals are to evaluate 1) the cellular mechanisms of neoplastic transformation of epithelial cells due to chemical carcinogens; 2) cellular mechanisms of tumor promotion in initiated epithelial cells; 3) the regulatory role of retinoids in differentiation and cell proliferation; and 4) the interaction of tumor cells with their environment. The work is organized into five projects.

The surprising finding that control and transformed rat liver-derived cells were shown to have different patterns of incorporation of the basement membrane components, laminin and fibronectin, into their matrices prompted us to investigate the specific effects of individual basement membrane components on cell behavior, especially cell growth and spreading. This was done by seeding sublines of control and transformed liver cells on coverslips coated either with fibronectin, laminin or type IV collagen and evaluating spreading by quantitative morphometric analysis and growth by measuring DNA and protein levels. The results show that laminin stimulates cell spreading in both control and transformed cells; however, fibronectin promotes maximal cell spreading only in transformed cells, thus providing evidence that transformation is accompanied by alterations in the sensitivity of the cells to basement membrane components. The striking capability of the transformed cells to spread and grow well, even on uncoated glass, and as a result, being less sensitive to defined substrates than the control sublines, demonstrates the high autonomy of these transformed cells.

Electron microscopic and immunofluorescence studies reveal that major factors for phenotypic differences between tumorigenic and control cells are indeed in the area of cell-cell and cell-substrate adhesion. The results of our studies give evidence, in cultures of transformed cells, for an increase in cell substrate adhesion due to increases in the number of focal contacts and in the expression of fibronectin; concomitantly, a loss of cell-cell adhesion via intermediate junctions is indicated. The changed adhesion patterns in the transformed cell cultures may be defined as progressive deficiencies in cell contact interactions. Although decreased cell-substrate adhesion is commonly associated with tumorigenic transformation (as has been shown for the RSV transformed cells), our contrasting observation of decreased cell-cell contact associated with increased cell-substrate adhesion in the transformed liver

epithelium makes this system a unique and valuable model for the study of the transformation process independent of a reduction in cell-substrate adhesion.

Promotion-dependent changes by the tumor promoter TPA on initiated mouse skin (line JB6) derived from promotable epithelial cells indicate that a single TPA exposure causes only a low percentage of these cells to become transformed. Moreover, TPA-induced morphologic alterations of both the cytoskeleton and the extracellular matrix are, to a large extent, reversible upon removal of the tumor promoter. To obtain malignant conversion with phenotypic alterations, however, both repeated cloning and repeated exposure of the cells to the promoter are necessary. Our morphologic studies were complemented by investigation of the H-ras oncogene product p21 to gain new insights into functional mechanisms of cell transformation. Our results provide a new finding suggesting a role of the cellular ras gene in tumor promotion. We found the H-ras oncogene product p21 present in focus-forming cells; yet, constant presence of the protein p21 was not necessary for the maintenance of the transformed state. The result suggests a link between the H-ras oncogene and promoter-dependent focus formation.

It has been proposed that the loss of contact inhibition and gap junctional communication has a significant role in tumor promotion, providing initiated cells with the means to escape from the controlling influence of their neighbors. Our study was extended to include examination of intercellular communication during tumor promotion. If an inhibitory effect of TPA on intercellular communication and gap junction formation could be detected in the JB6 cell system, it would be of great interest to determine whether the inhibitory effect is persistent in P⁺ cells and transient in P⁻ cells. We have studied the correlation between intercellular communication and TPA-promotion by observing ³H-uridine transfer. Our results, thus far only preliminary, give evidence that there exists only a limited correlation with respect to the loss of gap junctional communication during promotion when P⁺ and P⁻ cells of the JB6 systems are compared; however, NIH 3T3 cells, CHO cells and RT101 cells show, in the presence of TPA, a distinct loss of intercellular communication, indicating the importance of inherent characteristics of individual cell lines in this study.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP04542-14 LCC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Chemistry of N-Nitroso Compounds and Other Substances of Interest in Cancer Research		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: L. K. Keefer Chief, Chemistry Section LCC NCI Others: T. Anjo Visiting Associate LCC NCI H. S. Hu Staff Fellow LCC NCI J. Malin Guest Researcher LCC NCI Y.-H. Heur Visiting Fellow LCC NCI		
COOPERATING UNITS (if any) PRI, Frederick, MD (G. Lunn, L. Ohannesian, D. Williams, E. Sansone); SK&F Labs., Philadelphia, PA (B. Mico); NJ Med. Sch., Newark, NJ (C. Yang); Universitatsspital Zurich, Zurich, Switzerland (P. Kleihues and E. von Hofe); U. of Wash., Seattle, WA (T. Baillie)		
LAB/BRANCH Laboratory of Comparative Carcinogenesis		
SECTION Chemistry Section		
INSTITUTE AND LOCATION NCI, NIH, Frederick, MD 21701-1013		
TOTAL MAN-YEARS: 2.3	PROFESSIONAL: 2.3	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Mechanisms potentially responsible for formation, activation, and detoxication of carcinogenic N-nitroso compounds in the human body are under intensive investigation. The bifunctional amine, piperazine, which is used as an anthelmintic in humans, has been shown to react with nitrite with remarkable facility under catalysis by certain iron complexes. The results suggest that similar catalysis by biological iron compounds may be responsible for some endogenous formation of nitrosamines in vivo. Activation studies have revealed that many volatile anesthetics, including ether, act as competitive inhibitors of nitrosamine metabolism and that N-nitrosomethylethylamine can 2-hydroxyethylate as well as methylate and ethylate DNA. The latter result was predicted on the basis of deuterium isotope effect investigations. The deuterium isotope effect on conversion of formaldehyde and formate to carbon dioxide--one-carbon intermediates in the oxidation of N-nitrosodimethylethylamine (NDMA)--has been found to be negligible. The extent of denitrosation, a potentially very important detoxication route for NDMA, has been estimated in vivo from urinary methylamine excretion data. Refinements of destruction methods for carcinogenic N-nitroso compounds have been introduced and several salts of nitrosamines with strong acids have been prepared.		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

L. K. Keefer	Chief, Chemistry Section	LCC	NCI
T. Anjo	Visiting Associate	LCC	NCI
H. Hu	Staff Fellow	LCC	NCI
J. Malin	Guest Researcher	LCC	NCI
Y.-H. Heur	Visiting Fellow	LCC	NCI

Objectives:

Generally, to apply the methods and concepts of chemistry toward the solution of important problems in cancer research, especially by elucidating new mechanisms of formation, destruction, metabolism, and biological action of nitrosamines and related carcinogens. Specifically, (1) to establish mechanisms of nitrosamine formation so that strategies for preventing environmental contamination by these compounds can be developed; (2) to gather information on the chemistry of nitrosamine destruction so that procedures may be devised for intercepting these carcinogens before human exposure can occur; (3) to study the interactions between N-nitroso compounds and organisms exposed to them, with the aim of inferring ways of protecting victims of unavoidable nitrosamine exposure from their carcinogenic effects; (4) to characterize the fundamental physical and chemical properties of the carcinogenic N-nitroso compounds and other substances of interest in cancer research as a means of contributing to the general fund of knowledge about such materials.

Methods Employed:

The usual methods of organic and biological chemistry were employed, including gas and high pressure liquid chromatography, mass spectrometry, scintillation counting, and spectrophotometry.

Major Findings:

Concerning nitrosamine formation, we have found, in collaboration with D. Williams, that the human anthelmintic, piperazine, was nitrosated with extraordinary facility at pH ~ 10 under catalysis by certain iron complexes; this confirms predictions based on mechanisms studies with other amines and suggests that similar catalysis by metal-containing natural products might be responsible for some of the as-yet-unexplained endogenous formation of nitrosamines.

In studying the metabolic activation of nitrosamine carcinogens, we have learned, in collaboration with P. Kleihues and E. von Hofe, that N-nitrosomethylethylamine (NMEA) can serve as a 2-hydroxyethylating (as well as methylating and ethylating) agent in vivo. The existence of this phenomenon had been predicted from published data on the effects of β -deuteration of NMEA on organotropism in carcinogenesis by this compound. In collaboration with C. S. Yang, several volatile anesthetics, including ether, have been shown to be competitive inhibitors of nitrosamine metabolism. An assumption made in an earlier deuterium isotope effect study that N-nitrosodimethylamine (NDMA) metabolism can be reliably measured by following exhalation of labeled carbon dioxide regardless of whether the NDMA is deuterated or not, has been directly

tested in collaboration with T. Baillie. Substrate deuteration was found to have little or no effect on metabolism of either of the one-carbon intermediates, formaldehyde or formate, indicating that the previous assumption was valid. Regarding detoxication mechanisms for the N-nitroso compounds, first estimates of the extent of metabolic denitrosation of NDMA in the intact mammal have been made in collaboration with B. Mico. Using urinary excretion of methylamine (previously shown to be the basic organic product of the denitrosation reaction) as an index of *in vivo* denitrosation in the rat, the proportion of an injected NDMA dose that was eliminated by this presumably deactivating pathway was calculated to be about 10%.

In the nitrosamine chemistry studies, a patent has been obtained, in collaboration with G. Lunn, for a procedure to destroy dialkyl nitrosamines and related carcinogens in applications of potential commercial importance; and the method has been adapted to provide a general means of decomposing N-nitroso compounds during laboratory cleanup and waste disposal operations. Several crystalline salts of dialkyl nitrosamines with strong acids have been prepared in collaboration with L. Ohannesian, including some with stability and structural characteristics suggestive of fundamentally new bonding patterns.

Publications:

Keefer, L. K., Garland, W. A., Oldfield, N. F., Swagzdis, J. E. and Mico, B. A.: Inhibition of N-nitrosodimethylamine metabolism in rats by ether anesthesia. Cancer Res. 45: 5457-5460, 1985.

Kroeger-Koepke, M. B., Michejda, C. J., Roller, P. P. and Keefer, L. K.: Use of 3,4-dichlorobenzenethiol as a trapping agent for alkylating intermediates during in vitro metabolism of nitrosamines. Cancer Res. 45: 2973-2975, 1985.

Lunn, G., Sansone, E. B. and Keefer, L. K.: General cleavage of N-N and N-O bonds using nickel/aluminum alloy. Synthesis 12: 1104-1108, 1985.

Mico, B. A., Swagzdis, J. E., Hu, H. S.-W., Keefer, L. K., Oldfield, N. F. and Garland, W. A.: Low-dose in vivo pharmacokinetic and deuterium isotope effect studies of N-nitrosodimethylamine in rats. Cancer Res. 45: 6280-6285, 1985.

von Hofe, E., Grahmann, F., Keefer, L. K., Lijinsky, W., Nelson, V. and Kleihues, P.: Methylation versus ethylation of DNA in target and nontarget tissues of Fischer 344 rats treated with N-nitrosomethylethylamine. Cancer Res. 46: 1038-1042, 1986.

von Hofe, E., Kleihues, P. and Keefer, L. K.: Extent of DNA 2-hydroxyethylation by N-nitrosomethylethylamine and N-nitrosodiethylamine in vivo. Carcinogenesis (In Press)

Patents:

Keefer, L. K., and Lunn, G.

U.S. Patent 4,535,154. Reductive Destruction of Nitrosamines, Hydrazines, Nitramines, Azo- and Azoxy-Compounds, August 13, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP04580-12 LCC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) The Role of Lipotropes in Carcinogenesis		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: L. A. Poirier Chief, Nutrition and Metabolism Section LCC NCI Others: M. J. Wilson Chemist LCC NCI P. T. Allen Microbiologist LCC NCI		
COOPERATING UNITS (if any) Hotel-Dieu de Quebec, Quebec, Canada (L. Belanger); McArdle Laboratory, Madison, WI (H. Pitot)		
LAB/BRANCH Laboratory of Comparative Carcinogenesis		
SECTION Nutrition and Metabolism Section		
INSTITUTE AND LOCATION NCI, NIH, Frederick, Maryland 21701-1013		
TOTAL MAN-YEARS: 3.0	PROFESSIONAL: 2.0	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) The mechanisms responsible for the alteration of chemical carcinogenesis by the dietary lipotropes, choline, methionine, folic acid and vitamin B-12, have been studied. The metabolism and carcinogenic activity of ethionine in different species is being compared. Correlations between the tissue levels of the physiological methyl donor S-adenosylmethionine, its chief metabolic inhibitor, S-adenosylhomocysteine, and 5-methylcytosine in animals treated with carcinogens, liver tumor promoters and methyl-deficient diets are being determined. Using standard bioassays, the effects of (1) the length of time of dietary methyl deprivation, (2) the interaction between methyl deprivation and hepatocarcinogens, and (3) deficiencies of other essential nutrients on hepatocarcinogenesis are under investigation. The effects of carcinogens and methylase inhibitors on the general and specific gene hypomethylation in target tissues are examined.		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

L. A. Poirier	Chief, Nutrition and Metabolism Section	LCC	NCI
M. J. Wilson	Chemist	LCC	NCI
P. T. Allen	Microbiologist	LCC	NCI

Objectives:

To determine the mechanism(s) by which physiological methyl deprivation produces liver carcinomas. To determine the extent to which methyl deprivation contributes to carcinogenesis in extrahepatic tissues.

Methods Employed:

The effects of carcinogenesis in rodents of dietary regimes altering the in vivo bioavailability of the chief physiological methyl donor, S-adenosylmethionine, are investigated. The dietary components varied to include the methyl group-providing compounds, methionine and choline; the vitamins responsible for methyl group biosynthesis, folic acid and vitamin B₁₂; and the methionine antagonist, ethionine.

Both complete and two-stage carcinogenesis studies are employed. The early histological events associated with hepatocarcinogenesis are monitored by light microscopy using specialized stains. Tissue levels of S-adenosylmethionine, S-adenosylethionine, S-adenosylhomocysteine and 5-methyldeoxycytidine in DNA, are determined using appropriate combinations of HPLC and chromatographic systems developed in this Laboratory, as well as of standard spectrophotometric and radioisotopic techniques.

Serum levels of alphafetoprotein in rats undergoing carcinogenesis by methyl deprivation and the transfecting activity of DNA from tumors arising in methyl-deficient rats are determined by collaborative studies with other groups.

Major Findings:

The pursuit of this project has led to the major observation that dietary methyl deprivation alone causes liver cancer in male F344 rats and B6C3F1 mice. The chronic administration of methionine- and choline-deficient diets produced a high incidence of liver cancer in both initiated and uninitiated animals. Methyl deprivation, even in uninitiated rats, leads to the formation of preneoplastic lesions, such as enzymealtered foci and elevated levels of serum alphafetoprotein commonly seen during hepatocarcinogenesis by chemicals. In a recent study, a slight but significant elevation in the incidence of squamous papillomas has been seen in the forestomachs of rats fed a diet deficient in vitamin B₁₂ as well as methionine and choline. Dietary methyl deprivation induced a dedifferentiation of pancreatic acinar cells into hepatocyte-like cells.

In general, the biological effects of methyl deprivation and ethionine administration can be correlated with their biochemical effects in different tissues. For example, of all rat organs studied, the liver suffered the greatest decline in the

ratio of S-adenosylmethionine to S-adenosylethionine (in ethionine-fed rats) or to the physiological methylase inhibitor, S-adenosylhomocysteine (in choline- and methionine-deficient animals). In both cases such decreases were accompanied by a significant decline in the 5-methyldeoxycytidine content in hepatic DNA. The chronic administration of diets to C3H mice, which are sensitive to the hepatocarcinogenic effects of ethionine-containing diets to three strains of mice, produced ratios of hepatic S-adenosylethionine to S-adenosylmethionine. Also, the feeding of phenobarbital to ethionine-treated rats decreased the hepatocarcinogenic activity of the latter compound and led to decreased ratios of S-adenosylethionine to S-adenosylmethionine in their livers. These results provide good evidence that dietary methyl insufficiency results in hypomethylated DNA and, at least under some conditions, plays a major role in hepatocarcinogenesis.

Publications:

Hoover, K. L., Hyde, C. L., Wenk, M. L. and Poirier, L. A.: Ethionine carcinogenesis in CD-1, BALB/c and C3H mice. Carcinogenesis (In Press)

Hoover, K. L. and Poirier, L. A.: Hepatocyte-like cells within the pancreas of rats fed methyl-deficient diets. J. Nutr. (In Press)

Poirier, L. A.: Brief history of the role of nutrition in carcinogenesis. In Poirier, L. A., Pariza, M. and Newberne, P. M. (Eds.): Essential Nutrients in Carcinogenesis. New York, Plenum Press (In Press)

Poirier, L. A.: The role of methionine carcinogenesis in vivo. In Poirier, L. A., Pariza, M. and Newberne, P. M. (Eds.): Essential Nutrients in Carcinogenesis. New York, Plenum Press (In Press)

Poirier, L. A.: Stages in carcinogenesis: Effects of diet. Am. J. Clin. Nutr. (In Press)

Poirier, L. A., Pariza, M. and Newberne, P. M. (Eds.): Essential Nutrients in Carcinogenesis. New York, Plenum Press (In Press)

Poirier, L. A., Wilson, M. J. and Shivapurkar, N.: Hepatocarcinogenesis and DNA hypomethylation in methyl-deficient animals. In Borchardt, R. (Ed.): The Biochemistry of S-adenosylmethionine as a Basis for Drug Design. Clifton, NY, Humana Press (In Press)

Shivapurkar, N., Hoover, K. L. and Poirier, L. A.: Effect of methionine and choline on liver tumor promotion by phenobarbital and DDT in diethylnitrosamine-initiated rats. Carcinogenesis 7: 547-550, 1986.

Shivapurkar, N., Wilson, M. J., Hoover, K. L., Mikol, Y. B., Creasia, D. and Poirier, L. A.: Hepatic DNA methylation and liver tumor formation in male C3H mice fed methionine- and choline-deficient diets. JNCI (In Press)

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP04582-11 LCC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Mechanisms of Inorganic Carcinogenesis: Nickel		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: K. S. Kasprzak Visiting Scientist LCC NCI		
Others: L. A. Poirier Chief, Nutrition and Metabolism Section LCC NCI M. P. Waalkes Senior Staff Fellow LCC NCI J. M. Ward Chief, Tumor Pathology & Pathogenesis Section LCC NCI C. W. Reynolds Head, Cell. & Mol. Immunol. Section LEI NCI A. C. Denn Biologist LEI NCI D. Reichardt Biologist LEI NCI		
COOPERATING UNITS (if any) Program Resources, Inc., Frederick, MD (O. Weislow, H. Issaq, R. Kovatch, C. Riggs); Microbiological Associates, Inc., Bethesda, MD (M. Wenk)		
LAB/BRANCH Laboratory of Comparative Carcinogenesis		
SECTION Inorganic Carcinogenesis Working Group, Office of the Chief		
INSTITUTE AND LOCATION NCI, NIH, Frederick, Maryland 21701-1013		
TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Inhibitory effects of the essential divalent metals, magnesium and zinc, on the carcinogenicity and acute toxicity of a divalent metal carcinogen, nickel, have been investigated in carcinogenesis, toxicity, and biochemical studies. Magnesium has been found to counteract nickel carcinogenesis in rat muscle in a dose-dependent mode. It prolongs the latent period and substantially decreases the incidence of nickel-induced sarcomas, while zinc prolongs the latency of tumors without significant effect on their incidence. The inhibitory action of magnesium is strictly local; that of zinc is systemic. Magnesium greatly attenuates the post-injection necrosis and inflammation, particularly the immediate infiltration of neutrophils into the site of injection. Similarly in mice, i.p. injections of nickel cause a vivid infiltration of inflammatory cells that can be inhibited by a simultaneous injection of magnesium. Neither magnesium nor nickel, alone or combined, have any effect on natural killer cell activity in rat blood and injected muscle. Magnesium and zinc appear to be equally effective in antagonizing acute toxicity of nickel, but their individual mechanisms of action are different. Magnesium affects pharmacokinetics, while zinc affects the pharmacodynamics of nickel in rats. Likewise different are the two physiological metals in inhibiting nickel-DNA interactions in vitro. Further exploration of the mechanisms of nickel interactions with magnesium and zinc with more emphasis on the role of all three metals in the mediation of local inflammatory reactions is under way.		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

K. S. Kasprzak	Visiting Scientist	LCC	NCI
L. A. Poirier	Chief, Nutrition and Metabolism Section	LCC	NCI
M. P. Waalkes	Senior Staff Fellow	LCC	NCI
J. M. Ward	Chief, Tumor Pathology & Pathogenesis Section	LCC	NCI
C. W. Reynolds	Head, Cell. Mol. Immunol. Section	LEI	NCI
A. C. Denn	Biologist	LEI	NCI
D. Reichardt	Biologist	LEI	NCI

Objectives:

To study the mechanisms of nickel carcinogenesis by investigating a hypothesis that nickel and possibly other metal carcinogens act, at least in part, through interactions with the essential divalent metals, cadmium, magnesium, zinc, iron, and others. Uncoding the molecular nature of these interactions would provide some new insights into the mechanisms of carcinogenesis and eventually open up prospects of practical application of the essential metals in cancer prevention and treatment.

Methods Employed:

The carcinogenic and toxic activities of nickel in the presence of variable amounts of magnesium or zinc have been studied in vivo and in vitro using standard protocols. These include various-term injections of the carcinogenic nickel compounds alone, or combined with the physiological metal salt, into rats or mice followed by examination at necropsy for morphological and biochemical changes. Metabolism of nickel compounds in rats and mice and the influence of magnesium and zinc upon this metabolism as well as upon the biochemical effects of nickel toxicity at the organismal, tissue, and cellular levels, are determined by radioisotopic, standard analytical, and immunohistochemical methods.

Major Findings:

Inhibition of nickel carcinogenesis by magnesium involves anti-necrotic and anti-inflammatory action at the site of i.m. nickel injection. The anti-carcinogenic effects depend on the dose of magnesium and are of strictly local character. Magnesium prolongs the latent period and decreases the final yield of nickel-induced sarcomas. Only water-insoluble magnesium carbonate applied locally is effective. Highly soluble magnesium acetate has no effect whatsoever. Serial histologic examination of the anti-inflammatory action of magnesium at the injection site reveals a marked decrease in the number of infiltrating neutrophils and an increase in the abundance of macrophages, compared with rats given nickel alone. Likewise, nickel subsulfide, injected i.p. to mice, induces a vivid infiltration of inflammatory cells that can be greatly inhibited by a simultaneous injection of magnesium carbonate. Based on previous findings in mice, it was predicted that nickel might also impose its action by suppressing the activity of the natural killer (NK) cells and that magnesium might reverse this effect. However, it appeared that the NK cell activity (Cr-51 release) in rats could not be altered by these metals. Activity of NK in peripheral blood and spleen of rats, 1-21 days after treatment with i.m. nickel

and/or magnesium, up to the maximum tolerated doses, did not differ significantly from that in control rats. Similarly, immunohistologic staining (OX-8) revealed the presence of viable NK cells in muscles adjacent to the injected nickel subsulfide particles, with no apparent difference when magnesium had also been administered.

To establish a broader mechanistic base for further studies of nickel-magnesium interactions, the influence of magnesium on the acute toxicity of nickel was studied in rats, including effects on mortality, nickel pharmacokinetics, hyperglycemia, lipid peroxidation, cytochrome P-450, and ATPase activity. Magnesium significantly decreased the lethality of nickel, increased nickel excretion, and diminished nickel uptake in the kidneys, lung, and liver. No effects of magnesium were found upon nickel-induced hyperglycemia and increased lipid peroxidation in the liver and kidney. Hence, the antagonism of nickel toxicity by magnesium involves pharmacokinetic rather than pharmacodynamic mechanisms, most likely at the transmembrane transport level. In this respect magnesium differs substantially from zinc. The latter was found previously to antagonize nickel toxicity at some enzymatic, but not transport, levels. Magnesium and zinc also have very different capacities to inhibit nickel binding to DNA in vitro.

To complete the comparisons of the interaction modes of magnesium and zinc with nickel, the effects of zinc upon nickel carcinogenesis have been studied in rats. The animals were injected i.m. with nickel subsulfide and zinc oxide or acetate at the same place or separately. The results indicate that zinc markedly prolongs the latent period of tumor induction with only a slight effect on the final tumor yield in 1.5 years. Both water-soluble and insoluble zinc salts, administered either locally or systemically, are effective. Analysis of the injection site revealed a long retention of zinc ($T_{1/2} = 30$ days), irrespective of the water solubility of its injected salt. Also, zinc exerted no influence on the retention of nickel in the rat muscle during the first month post injection. The antagonism of nickel by magnesium apparently has a different molecular basis than antagonism by zinc.

Further exploration of the sites and mechanisms of nickel interactions with magnesium and zinc with more emphasis on the inflammation-mediating role of all three metals is under way.

Publications:

Kasprzak, K. S. and Poirier, L. A.: Effects of calcium and magnesium on nickel(II) uptake and stimulation of thymidine incorporation into DNA in the lungs of strain A mice. Carcinogenesis 6: 1819-1821, 1985.

Kasprzak, K. S., Quander, R. V. and Poirier, L. A.: Effects of calcium and magnesium salts on nickel subsulfide carcinogenicity in Fischer rats. Carcinogenesis 6: 1161-1166, 1985.

Kasprzak, K. S., Waalkes, M. P. and Poirier, L. A.: Antagonism by essential divalent metals and amino acids of nickel(II)-DNA binding in vitro. Toxicol. Appl. Pharmacol. 82: 336-343, 1986.

Kasprzak, K. S. and Waalkes, M. P.: Role of calcium, magnesium and zinc in carcinogenesis. In Poirier, L. A., Pariza, M. and Newberne, P.M. (Eds.): Essential Nutrients in Carcinogenesis. New York, Plenum Press (In Press)

Kasprzak, K. S., Waalkes, M. P. and Poirier, L. A.: Effects of essential divalent metals on carcinogenicity and metabolism of nickel and cadmium. Biol. Trace Element Res. (In Press)

Kasprzak, K. S., Waalkes, M. P. and Poirier, L. A.: Effects of magnesium acetate on the toxicity of nickelous acetate in rats. Toxicology (In Press)

Williams, D. F., Natiella, J. R., Lucas, L. C., Avery, J. K., Cox, C., Goldberg, A., Kasprzak, K. S., Millard, H. D., Rhyne, R. and Rupp, N.W.: Local tissue reactions. Carcinogenesis. In Lang, B. R., Morris, H. F. and Razzog, M. E. (Eds.): Biocompatibility, Toxicity and Hypersensitivity to Alloy Systems Used in Dentistry. Ann Arbor, Univ. of Michigan Press, 1986, pp. 247-276.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP04680-16 LCC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Application of In Vitro Systems to Study Perturbations of Methyl Metabolism		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	M. J. Wilson Chemist	LCC NCI
Others:	L. A. Poirier Chief, Nutrition and Metabolism Section	LCC NCI
	U. I. Heine Chief, Ultrastructural Studies Section	LCC NCI
	J. L. Junker Staff Fellow	LCC NCI
	S. Rehm Visiting Associate	LCC NCI
	D. G. Blair Chief, Microbiology Section	LMO NCI
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Comparative Carcinogenesis		
SECTION Nutrition and Metabolism Section		
INSTITUTE AND LOCATION NCI, NIH, Frederick, Maryland 21701-1013		
TOTAL MAN-YEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Epithelial cells derived from the livers of 10-day-old Fischer 344 rats are used as a model system for studying the mechanism of carcinogenesis resulting from an insufficiency of methyl donors. Transformation of liver cells has been achieved following treatment with 3-deazaadenosine (DAA). This compound is metabolized to 3-deazaadenosylhomocysteine, a potent inhibitor of S-adenosylhomocysteine (AdoHcy) hydrolase, and results in an accumulation of AdoHcy, a competitive inhibitor of most physiological methylation reactions. DNA has been isolated from tumors induced in rats initiated with DEN and fed a diet deficient in methionine and choline and used in the NIH 3T3 cell transfection assay. Results indicate that activation of the c-Ha-ras oncogene appears to be involved in the development of hepatocellular carcinomas in methyl-deficient rats. This gene is hypomethylated in the liver tumors of rats fed the methyl-deficient diets.		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

M. J. Wilson	Chemist	LCC	NCI
L. A. Poirier	Chief, Nutrition and Metabolism Section	LCC	NCI
U. I. Heine	Chief, Ultrastructural Studies Section	LCC	NCI
J. L. Junker	Staff Fellow	LCC	NCI
S. Rehm	Visiting Associate	LCC	NCI
D. G. Blair	Chief, Microbiology Section	LMO	NCI

Objectives:

To determine the mechanism(s) of neoplastic transformation induced by antagonists of methyl metabolism using a cell culture model system. Specific goals are: 1) to establish the suitability of rat liver cells in culture as a model for studying the mechanism of carcinogenicity by antagonists of methyl metabolism; 2) to determine the potential carcinogenicity of known inhibitors of methylation reactions; 3) to examine the effect of these compounds on DNA methylation in order to investigate hypomethylation of DNA as a possible mechanism of transformation; and 4) to determine the transforming potential, by transfection into NIH 3T3 cells, of DNA isolated from hepatocellular carcinomas induced by methyl-deprivation and to determine the onco-gene(s) responsible for such transformation as well as the possible role of hypomethylation on its activation.

Methods Employed:

Rat liver cells (TRL 1215) were treated with DAA for up to 12 weeks then maintained in culture in the absence of DAA. Tumorigenicity and anchorage-independent growth were monitored at monthly intervals. The percentage of cytosine residues modified to 5-methylcytosine are determined in TRL 1215 cells undergoing treatment with DAA at doses known to induce transformation. DNA is isolated, purified, subjected to enzymatic hydrolysis and the hydrolysate chromatographed on HPLC. DNA is isolated from hepatocellular carcinomas produced in F344 rats following prolonged dietary deprivation of methyl donors with and without prior initiation with DEN (20 mg/kg body weight). The average molecular weight of isolated DNAs, determined by agarose gel electrophoresis, is greater than 50 kilobases. DNA transfections of NIH 3T3 cells were then performed. The cultures are scored for morphologically transformed foci after 23 days. High molecular weight DNA is isolated from primary transformants, used in a second cycle of transfection and secondary transformants isolated.

Major Findings:

Tumors developed in a fraction of animals injected with cells treated for up to 12 weeks with 0.075 or 0.100 mM deazaadenosine. Tumor incidence increased when cells were maintained in culture for an additional 1 or 2 months in the absence of deazaadenosine. Growth in soft agar was also observed at these time points. Tumors were not present in animals injected with cells treated with 0.150 mM deazaadenosine for the experimental period described. The control cells have remained negative throughout this experiment. The level of DNA methylation in cells treated with

0.075 mM deazaadenosine for 1 month was 38% less than that of untreated cells. A similar level of suppression of methylation was observed in cells treated for 3 months.

High molecular weight DNA isolated from liver tumors induced in rats by DEN initiation, followed by methyl-deprivation, has produced transformed foci in the NIH 3T3 cell transfection assay. Approximately 12% of tumor DNAs tested (a total of three) were positive while all control DNAs have been negative. The presence of rat DNA in the primary transformants thus far examined has been demonstrated using a probe specific for rat repetitive sequences. The data obtained indicate the presence of an activated c-Ha-ras gene in the DNA of hepatocellular carcinomas from rats resulting from the exposure to a carcinogenic regimen. Initial studies with restriction enzymes indicate that this gene is hypomethylated.

Publications:

Junker, J. L. and Wilson, M. J.: Divergent expression of laminin and fibronectin in nontumorigenic and transformed liver epithelial cells. J. Cell Sci. 76: 213-223, 1985.

Waalkes, M. P., Wilson, M. J. and Poirier, L. A.: Reduced cadmium-induced cytotoxicity in cultured liver cells following 5-azacytidine pretreatment. Toxicol. Appl. Pharmacol. 81: 250-257, 1985.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP04812-18 LCC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Cell Interactions During Transformation of Epithelial Cells		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: U. I. Heine Chief, Ultrastructural Studies Section LCC NCI Others: J. L. Junker Staff Fellow LCC NCI M. J. Wilson Chemist LCC NCI H. Miki Visiting Fellow LCC NCI		
COOPERATING UNITS (if any) Biological Products Laboratory, Program Resources, Inc., Frederick, MD (E. F. Munoz)		
LAB/BRANCH Laboratory of Comparative Carcinogenesis		
SECTION Ultrastructural Studies Section		
INSTITUTE AND LOCATION NCI, NIH, Frederick, Maryland 21701-1013		
TOTAL MAN-YEARS: 2.5	PROFESSIONAL: 2.0	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> In order to characterize cell-cell and cell-substrate adhesion changes that are critical factors in the regulation of growth and development, we have employed two model systems: a) ethionine-transformed rat liver epithelial cells (line TRL 1215) for which controls at low and high passage level are available, and b) mouse epidermis-derived promotable cells of line JB6. By using immunofluorescence-labeled probes against major matrix proteins, we established that transformation in liver cells is accompanied by increased cell-substrate adhesion and cell spreading, thereby showing, in this cell system, that the capability of anchorage-independent growth is not related to loss of cell-substrate adhesion. Extension of this study includes investigations in the modulation of cell behavior by individual components of the basement membrane such as laminin, fibronectin, and collagen. Results of this investigation provide evidence of increased sensitivity to fibronectin by the transformed cells, but more strongly demonstrate the autonomy of the transformed cells relative to the controls with respect to growth and attachment. </p> <p> To define the role of gap junctional communication in tumor promotion, nonpromotable, promotable and transformed epidermis-derived cells of line JB6, untransformed and transformed liver cells (TRL 1215), and NIH 3T3 cells were subjected to the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) and cell communication was measured by transfer of 3H-uridine. Our study suggests that TPA inhibits intercellular communication only among tumorigenic JB6 cells that overgrow surrounding cells; promotable and nonpromotable initiated cells are not distinguished by this protocol. Inhibition of uridine transfer as a short-term assay for tumor promoters thus gives a false negative in this cell line and results of the assay in other lines should be interpreted with caution. </p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

U. I. Heine	Chief, Ultrastructural Studies Section	LCC	NCI
J. L. Junker	Staff Fellow	LCC	NCI
M. J. Wilson	Chemist	LCC	NCI
H. Miki	Visiting Fellow	LCC	NCI

Objectives:

- 1) To characterize the changes in cytoskeletal, cell-cell and cell-matrix interactions which are indicative of epithelial cell transformation, using as model systems a) ethionine transformed liver epithelial cells and b) promotable epidermis-derived cells of line JB6 together with appropriate controls.
- 2) To examine the effects of basement membrane components on cell structure and behavior as they relate to cell transformation.
- 3) To evaluate inhibition of intercellular communication by tumor promoters as a mechanism for tumor promotion.

Methods Employed and Major Findings:

Cell-cell and cell-substrate adhesion are critical factors in the regulation of growth and development. Thus, in order to understand tumor promotion, malignant conversion and unrestrained growth, it is necessary to determine how a cell's interactions with surrounding cells and matrix are altered during transformation. It has been established in this laboratory that the liver-derived TRL 1215 cells are epithelial cells in which transformation after exposure to DL-ethionine is accompanied by increased cell-substrate adhesion and cell spreading, thereby showing, in this cell system, that there does not exist a direct linkage between capability for anchorage-independent growth and a loss of cell-substrate adhesion. By employing immunofluorescence-labeled probes against major matrix proteins, we established that enhanced cell-substrate adhesion is indicated by increased numbers of actin stress fibers, fibronectin fibers and adhesion plaques in ethionine-transformed cells. Yet, these transformed cells form multilayered foci and are fully capable of anchorage-independent growth and can produce tumors in syngeneic animals. In a continuing examination of cell-matrix interactions, a study of the modulation of cell behavior by individual components of the basement membrane was undertaken. Cells were grown on dishes coated with laminin, fibronectin or type IV collagen. Growth was determined using DNA and protein analyses. Cell spreading, an indicator of adhesion, was determined quantitatively by morphometric analysis of projected cell areas. The results of the study provide the following data: 1) laminin substrata increase the spreading and growth of both transformed and untransformed sublines, 2) a transformation-associated increase in sensitivity to fibronectin was observed, 3) laminin plays a role in retarding the overgrowth of postconfluent cells, and 4) the absolute values for spreading and growth are highest for the ethionine-transformed cells. However, proportionally, the transformed cells are less sensitive to the defined substrata than the control sublines, with the exception of the spreading response to fibronectin. Thus, the data emphasize the autonomy of the transformed cells and

show that their ability to spread and grow is less dependent on exogenous factors. This autonomy is also shown by growth of the three sublines in serum-free medium. Control cells have limited survival in serum-free medium; however, ethionine transformed cells are capable of proliferation in such a medium.

It has been proposed that the loss of gap junctional communication plays a significant role in tumor promotion, providing initiated cells with further means to escape from the controlling influence of their surrounding normal neighbors. We have studied the effect of 12-O-tetradecanoylphorbol-13-acetate (TPA) on intercellular communication in nonpromotable (C1 30), promotable (C1 41) and tumorigenic transformed (RT 101) mouse epidermal cells of line JB6, normal and transformed rat liver cells (TRL 1215), and NIH 3T3 cells. Monolayered donor cells were labeled with 5 μ Ci/ml of (3 H) uridine for 3 h and cocultured with recipient cells for 3 h with or without TPA. Cells were fixed with 2.5% glutaraldehyde in cacodylate buffer and processed for autoradiography. Nonpromotable, promotable, and tumorigenic JB6 cell lines have good intercellular communication under normal growth conditions. In the presence of TPA, RT 101 lose their intercellular communication both with themselves and with cells of C1 30 and C1 41. However, intercellular communication between C1 30 cells, between C1 41 cells, and between C1 30 and C1 41 cells was not significantly blocked by TPA. The intercellular communication of normal and transformed liver cells was not significantly inhibited by TPA, either. On the other hand, TPA inhibited intercellular communication between both CHO and NIH 3T3 cells. Our results, with respect to JB6 cells, suggest that TPA allows only tumorigenic cells to overgrow the surrounding cells by inhibiting intercellular communication. The negative results obtained with C1 30, C1 41, and liver cells and the positive results with CHO and NIH 3T3 cells give an indication of the importance of specific and inherent characteristics of the individual cell lines in this protocol.

Publications:

Junker, J. L. and Wilson, M. J.: Divergent expression of laminin and fibronectin in nontumorigenic and transformed liver epithelial cells. J. Cell Sci. 76: 213-223, 1985.

Takahashi, K., Heine, U. I., Junker, J. L., Colburn, N. H. and Rice, J. M.: The role of cytoskeleton changes and expression of the H-ras oncogene during promotion of neoplastic transformation in mouse epidermal JB6 cells. Cancer Res. (In Press)

Ward, J. M., Pardue, R. L., Junker, J. L., Takahashi, K., Shih, T. Y. and Weislow, O. S.: Immunocytochemical localization of Ras-Ha-p21 in normal and neoplastic cells in fixed tissue sections from Harvey sarcoma virus-infected mice. Carcinogenesis 7: 645-651, 1986.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05092-08 LCC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Transplacental Carcinogenesis and Tumor Promotion in Nonhuman Primates		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: A. E. Palmer Research Veterinarian LCC NCI Others: J. M. Rice Chief LCC NCI J. M. Ward Chief, Tumor Pathol. and Pathogen. Section LCC NCI L. M. Anderson Expert LCC NCI P. J. Donovan Chemist LCC NCI A. O. Perantoni Microbiologist LCC NCI		
COOPERATING UNITS (if any) Meloy Laboratories, Inc., Rockville, MD (J. Phillips); Medical College of Wisconsin, Milwaukee, WI (R. Hussa)		
LAB/BRANCH Laboratory of Comparative Carcinogenesis		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Frederick, Maryland 21701-1013		
TOTAL MAN-YEARS: 3.2	PROFESSIONAL: 2.0	OTHER: 1.2
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Nonhuman primates of the species <u>Erythrocebus patas</u> (patas), <u>Macaca fascicularis</u> (cynomologus) and <u>Cebus apella</u> (cebus), are subjected to direct-acting or metabolism-dependent chemical carcinogens by transplacental or direct exposure. In some cases the carcinogen-treated animals are subsequently exposed to chemicals that promote the development of neoplasms in rodents. Mechanisms of organ and species differences in the effects of chemical carcinogens and tumor promoters among rodent and nonhuman primate species are investigated. Induced tumors are evaluated by light microscopy using standard staining procedures, histochemical techniques and electron microscopy and are assayed for in vitro cultivability and transplantability to rodents. Selected tumors are subjected to DNA extraction and attempts are made to transfect NIH 3T3 cells with their DNA. These studies have shown that intrinsic susceptibility to transplacental carcinogenesis is greatest in nonhuman primates early in gestation and have provided the only animal model of chemically inducible gestational choriocarcinoma.		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

A. E. Palmer	Research Veterinarian	LCC	NCI
J. M. Rice	Chief	LCC	NCI
J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI
L. M. Anderson	Expert	LCC	NCI
P. J. Donovan	Chemist	LCC	NCI
A. O. Perantoni	Microbiologist	LCC	NCI

Objectives:

To study and characterize the variable sensitivities of different organ systems in nonhuman primates to carcinogens which act directly or require in vivo metabolism for carcinogenic activity during the prenatal and postnatal periods. To attempt to demonstrate the phenomenon of tumor promotion in nonhuman primates and to determine whether cell and tissue specificities of tumor-promoting chemicals demonstrated in rodents are similar in nonhuman primate models. To explore the activation of oncogenes in the course of chemical carcinogenesis in nonhuman primates.

Methods Employed:

Carcinogenic chemicals specifically selected or designed for a given study are purified after purchase or synthesized de novo and characterized thoroughly by chromatographic and spectroscopic procedures. Radiolabeled compounds are similarly prepared as required. The carcinogens are administered in precise doses to nonpregnant or exactly timed-pregnant nonhuman primates (patas or cynomolgus monkeys) and the treated animals and their offspring are followed carefully for the development of tumors. Agents which previously have been demonstrated to promote tumors in rodents are similarly purchased, purified or synthesized de novo and administered to primates after completion of an initiating regimen of exposure to organ-specific carcinogens.

Tumor-bearing nonhuman primates are intensively monitored to study tumor growth, body weight and clinical pathological changes. Selected animals are evaluated for tumor markers such as alphafetoprotein and chorionic gonadotropin. Selected tumors which may cause suffering are carefully evaluated on an individual basis and may be surgically removed to reduce suffering and to prolong life. When tumors are judged to be inoperable and to be causing suffering or threatening life, animals are killed by euthanasia. Thorough gross postmortem examinations are performed and all gross lesions plus sections from all major organs are evaluated by light microscopy. Selected neoplasms are evaluated by electron microscopy and/or are cultured in vitro, then transplanted to athymic (nu/nu) mice. Selected tumors are subjected to DNA extraction and attempts are made to transfect NIH 3T3 cells with their DNA.

Major Findings:

Studies on carcinogenesis by ethylnitrosourea (ENU) and diethylnitrosamine (DEN) in *Erythrocebus patas*, an Old World monkey, continue and have been expanded to include aflatoxin B₁ (AFB₁). Additional cases of mesenchymal and epithelial tumors were observed in the offspring of monkeys that received ENU intravenously during

pregnancy, especially when exposure occurred during the first trimester of gestation. These additional findings support the tentative conclusions drawn previously that, like rodents, this species of nonhuman primate is quantitatively more susceptible to the direct-acting alkylating agent, ENU, during prenatal life, with animals exposed in utero exhibiting a higher incidence of tumors after a shorter latency than juvenile or adult animals that received a similar dose directly. Neoplasms continue to develop in primates now as old as 126 months (middle aged adults) that were exposed to low doses of ENU transplacentally during the first trimester of gestation (30 to 60 days).

Tumors induced in transplacentally exposed patas monkey include both embryonal and adult cellular types. Embryonal tumors have occurred at low frequency and have included nephroblastomas and rapidly-growing, lethal primary sarcomas of the lung. Patas monkeys treated transplacentally, which survive to adulthood, and those exposed directly to ENU have developed multiple bronchioalveolar adenomas of the lung, suggesting a biphasic response of this monkey's lungs to this carcinogen.

A total of nine female patas monkeys have died from a widely disseminated, hemorrhagic malignancy less than 7 months after the termination of a pregnancy during which they received intravenous ENU. The tumor has been characterized by small primary uterine lesions with metastatic lesions, primarily to the lungs. Morphologically the tumor cells in these cases resemble cytotrophoblast and are rapid-growing, causing death by hemorrhage. Tumor cells were found within placenta in one case and were usually widely disseminated within uterine and pulmonary vessels. One additional animal developed a relatively slow growing tumor of the uterus which invaded the pelvic organs. This tumor was predominantly of cytotrophoblast type, but also contained syncytiotrophoblast-like cells. Attempts to identify chorionic gonadotropins by serological means, using antisera that are reactive with the chorionic gonadotropins of man, the great apes, macaque monkeys, and several New World monkeys, were unsuccessful. However, biological activity has been identified in sera from pregnant patas monkeys using the mouse testicular interstitial cells in vitro. This activity occurs only between days 19 and 34 of gestation. Sera collected from five females with the rapidly fatal tumor at 0 to 47 days antemortem were tested by this biological test and all were negative. However, the serum from the animal with the tumor containing syncytiotrophoblast contained significant activity beginning 167 days antemortem, and the activity levels increased until the time of her death.

These observations provide the first chemically inducible animal model for gestational choriocarcinoma and suggest that chemicals may play a role in the development of this malignancy in humans, since incidence differs markedly throughout the world. Incidence in the human disease is highest in southeastern Asia where the chance of exposure to naturally occurring carcinogens, such as aflatoxin F₁ (AFB₁), is high. For this reason, studies are underway to investigate the carcinogenic activity of AFB₁ in the pregnant patas monkey.

In studies to demonstrate the phenomenon of tumor promotion in nonhuman primates, the liver is being used as the target organ. DEN, the most effective chemical carcinogen in nonhuman primates is used as an initiator and to date, the barbiturates, phenobarbital (PB) and sodium barbitol, are under study as promoters. Patas monkeys treated with DEN by the intravenous, transplacental and intraperitoneal routes were later subjected to daily doses of 15 mg/kg of PB in their drinking water. Tumor promotion occurred only in the liver and was evident regardless of the route of DEN

exposure and despite an interval of approximately 48 months after DEN given intravenously and transplacentally before the PB was started. Studies are underway, using the cynomolgus monkey (Macaca fascicularis), to investigate possible promoter activity by PB and sodium barbital.

Liver cytochrome P-450 and aminopyrine demethylase were measured in patas monkeys which received either DEN only or DEN plus 275 days of PB at 15 mg/kg/day. Levels of cytochrome P-450 were 3.1-fold and aminopyrine demethylase 3.6-fold higher in the PB recipients.

Publications:

None

Patents:

None

CONTRACTS IN SUPPORT OF THIS PROJECT

MELOY LABORATORIES, INC. (N01-CP-41016)Title: Resources for Transplacental Carcinogenesis in PrimatesCurrent Annual Level: \$310,045Man Years: 3.2Objectives:

This contract provides animal care and technical support for a colony of 185 animals. This project is designed to demonstrate and characterize transplacental carcinogenesis in nonhuman primates, especially the Erythrocebus patas, an Old World monkey. Additionally, related phenomena are studied, including the increased risk of carcinogenesis in adult females exposed to chemicals during pregnancy, tumor promotion, and mechanisms of cell and organ specificities and of species differences in the effects of both chemical carcinogens and tumor promoters.

Major Contributions:

Models for chemically inducible gestational choriocarcinoma and for pre-eclamptic toxicity of pregnancy have resulted from studies performed under this contract. Ethylnitrosourea (ENU) has been shown to be a potent carcinogen in the patas and the rhesus (Macaca mulatta) monkeys. In both species the fetus is more susceptible than is the adult, and this susceptibility is more pronounced during the first and early second trimesters of pregnancy. However, the kinds of tumors seen in the two species differ in their characteristics and distribution.

Diethylnitrosamine (DEN) given to pregnant patas monkeys during gestation did not cause tumors in the offspring or mothers after 4 years of observation. However, after 24 to 30 months of subsequent daily doses of phenobarbital comparable to therapeutic anticonvulsant levels in man, both offspring and mothers developed hepatocellular adenomas and carcinomas. Phenobarbital clearly can promote hepatocarcinogenesis in this species as it does in rats.

Except for the association between in utero exposure to diethylstilbestrol and the increased risk of vaginal adenocarcinoma during early adulthood, there is little known concerning the effects of carcinogens on the human fetus. Transplacental chemical carcinogenesis studies have been limited to rodent species which differ greatly from man. Most significant is the more rapid rate of fetal and neonatal growth and maturation in rodents. Nonhuman primates also have shorter gestations and mature more rapidly than do humans, but they are more similar to man in fetal growth, placentation and early development than are rodents. Some tumors induced to date in rhesus and patas monkeys by transplacental exposure to carcinogens resemble some congenital tumors or tumors of infancy and childhood seen in man, suggesting that prenatal exposure of humans to chemicals may be a factor in tumor incidence. The demonstration of tumor promotion in nonhuman primates provides significant evidence of the importance of this phenomenon to man.

Proposed Course:

Animals previously exposed to carcinogens will continue to be closely monitored for tumor development, and all tumors will be intensively studied. In addition, studies to demonstrate, more precisely, the varying sensitivity of the fetus during gestation are under way. Limited numbers of animals will be treated with agents known to be promotive in rodents, after limited transplacental exposure to carcinogens. The transplacental effects of chemicals other than ENU and DEN will be explored. The direct and transplacental carcinogenic effects of aflatoxin B₁ will be studied. This study will be combined with the companion Contract N01-CP-25613 for recompetition.

MELOY LABORATORIES, INC. (N01-CP-25613)

Title: Tumor Promotion in Cynomolgus Monkeys (Macaca fascicularis)

Current Annual Level: \$137,692

Man Years: 1.5

Objectives:

This contract provides animal care and technical support for a colony of 100 animals. This project is intended to demonstrate the phenomenon of tumor promotion in cynomolgus monkeys and to explore the promotive activity in this species of several chemicals known to promote tumors in rodents. The liver model was chosen because diethylnitrosamine (DEN) has been studied extensively and shown to be a predictable hepatocarcinogen in this species. Preliminary findings suggest that DEN initiates patas monkey liver when given intravenously or transplacentally. The effect of promoter compounds in vivo on liver metabolism, morphology and enzyme induction will be studied.

Major Contributions:

Preliminary studies indicate that the cynomolgus monkey liver is less responsive to the peroxisome proliferator Wyl4643 and to the chlorinated hydrocarbon alpha hexachlorocyclohexene than in the F344 rat liver. Monkeys treated daily with phenobarbital and barbitol develop histologically demonstrable changes in their hepatocytes which are similar to the rat. Additionally, mixed function oxidase levels of phenobarbital and barbitol-treated monkeys are increased, although the levels are lower than that found in patas monkeys. To date, early preneoplastic foci and changes resulting from barbiturate effects have been found only in animals that received diethylnitrosamine followed by barbiturates. Tumors have not yet been found.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05093-08 LCC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) In Vitro Studies on Organ Specificity in Transplacental Carcinogenesis		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: J. M. Rice	Chief	LCC NCI
Others: P. Donovan		Chemist LCC NCI
A. Perantoni		Microbiologist LCC NCI
T. Enomoto		Visiting Fellow LCC NCI
COOPERATING UNITS (if any) Microbiological Associates, Inc., Bethesda, MD (M.L. Wenk); Program Resources, Inc., Frederick, MD (B. Diwan)		
LAB/BRANCH Laboratory of Comparative Carcinogenesis		
SECTION Perinatal Carcinogenesis Section		
INSTITUTE AND LOCATION NCI, NIH, Frederick, Maryland 21701-1013		
TOTAL MAN-YEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) The roles of morphogenetic differentiation in controlling the phenotypic expression of neoplastic transformation, the degree of malignancy of tumors, and the susceptibility of developing organs to carcinogenesis are studied using organ culture and tissue transplantation techniques, with current emphasis on the kidney. A defined medium for growth of rat and mouse ureteric bud epithelium in monolayer culture has been developed in which epidermal growth factor and selenium have proved essential and insulin, hydrocortisone, and transferrin have proved highly beneficial. The ability of transplacentally administered carcinogens to induce genotoxic damage in cells of embryos or fetuses exposed at different stages of gestation was determined for rat, mouse, and Syrian hamster. Cells were isolated from exposed embryos and gene mutations at two to three loci (resistance to ouabain and 6-thioguanine and to diphtheria toxin in the hamster) were assayed in vitro with simultaneous determination of survival ability. Organ specificity of induced gene mutation is being determined in embryonal cells isolated from organs of various species exposed in utero at comparable stages of gestation. A maximum level of mutation induction was found to be inducible by N-nitrosoethylurea at day 9 of gestation for mesenchymal cells of the Syrian hamster and at day 9-10 for the F344 rat.		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. M. Rice	Chief	LCC	NCI
P. J. Donovan	Chemist	LCC	NCI
A. O. Perantoni	Microbiologist	LCC	NCI
T. Enomoto	Visiting Fellow	LCC	NCI

Objectives:

To identify and characterize those aspects of morphogenetic differentiation that modify the consequences of prenatal exposure to chemical carcinogens, especially in the nervous and genitourinary systems. The ultimate objective is to elucidate the control of expression of the neoplastic phenotype in transformed cells. To devise and apply improved quantitative selective mutation systems to embryonal and fetal primary cells in culture from donors previously treated in utero with chemical carcinogens. To determine the time course of maximum sensitivity to induced gene mutation of cells from embryos or fetuses transplacentally exposed to carcinogens at different stages of gestation. To determine quantitative dose curves for transplacentally induced gene mutation by selected carcinogens. To determine the sensitivity of various species to metabolism-independent transplacental chemical carcinogens and to determine inter- and intra-litter variations in response. To determine the organ specificity in various species of gene mutations transplacentally induced by nitrosoethylurea. To apply in vitro transformation assays to cells isolated from embryos of different species treated transplacentally with chemical carcinogens. To correlate the above quantitatively determined in vitro parameters with transplacental tumorigenesis data.

Methods Employed:

Direct mutagenicity assay. Gravid animals are injected with different doses of agents at precise stages of gestation. Primaries are made from either the whole fetus or selected organs of different species. Cells are cultured for an experimentally determined expression time in either conventional or selective media. Cells are then seeded for determination of survival and treated with the selective agent including diphtheria toxin, 6-thioguanine or ouabain. Brain-derived cells or putative astrocytes are grown according to the method of Sellbrenner in 10% fetal calf serum. Keratinocytes are grown in MCDB 153 with low calcium serum in the same concentrations for the different species as before. Lung cells are grown in MCDB 153, formulation of Peale and Ham, which is a serum-free medium. Hamster liver failed to grow under all conditions and therefore we are now attempting to culture rat and mouse hepatocytes using different methodologies.

Renal differentiation. Short- and long-term cell and organ culture techniques are developed for tissue rudiments which are then characterized by histochemical, light microscopic, and ultrastructural techniques. Cultures of both normal fetal organ rudiments and selected tumors are utilized to explore the effects of morphogenetic differentiation and its induction on the behavior of tumors of undifferentiated cell type, including nephroblastic tumors of the kidney. Tumors are induced transplacentally or by direct treatment in experimental animals to

provide suitable material for study and are transplanted serially in appropriate recipients to develop standard, manipulable models for studies in vitro. Surgical procedures relating to tissue transplantation are adapted as necessary to study the capacity of various recipient sites to modify differentiation of selected transplantable tumors. Substances and tissues known to influence differentiation are combined with tumors and normal undifferentiated inducing tissues, and interactive events in the differentiation of normal and neoplastic tissues are characterized.

Major Findings:

Renal mesenchyme from 13 or 14 gestation day F344 rats or 11 or 12 gestation day C57BL/6 mice was explanted into Dulbecco's minimum essential medium with 10% fetal bovine serum, and subsequent culture passages were characterized. Cultured cells retained features of undifferentiated renal mesenchymal cells by morphologic and immunocytochemical criteria. Most notably, cells expressed vimentin and fibronectin; cytokeratin, laminin, and gamma-glutamyl transpeptidase expression were not observed. These results are consistent with characterizations of the undifferentiated fetal element and inversely related to the differentiated cells of the nephron, which originate in the mesenchymal component. Hybridomas have been generated against cultured mesenchymal cells and are to be tested against cultured cells derived from chemically induced renal mesenchymal tumors prior to attempts to identify determinants of renal differentiation.

Cocultivation experiments, using cultured rat or mouse renal blastema with cultured rat or mouse ureteric bud, have yet to yield definitive differentiation. Since the media developed for propagation of either fetal renal component excluded the other component, we are currently evaluating medium additives to obtain a milieu which will permit maintenance of bud and blastemal cells and allow differentiation of the blastemal component. We have found epidermal growth factor, a medium component essential for bud cell propagation, to be markedly inhibitory to blastemal cell differentiation at concentrations normally used. In addition, techniques that allow formation of three-dimensional structures from monolayer-cultured cells have been devised in efforts to fulfill the need for such a structure as described for other cultured tissues.

We have previously reported quantitative transplacental mutagenic dose curves for various carcinogens. The most potent was determined to be the polycyclic hydrocarbon DMBA. The two direct-acting alkylating agents, MNU and NEU, were also very active. MNU was about twice as potent a transplacental mutagen as NEU, but because of increased toxicity at the higher doses NEU is more effective in vivo. Dose curves were also concluded on MMS, EMS, BP, MC, DENA and urethane.

A quantitative study of the mutagenic effect of X-ray administered at day 12 of gestation was initiated. Point mutation in the somatic cells was detected by resistance to 6-thioguanine. Induction of 6-TG mutants per surviving cell increased in a linear fashion at doses below 200 R, but decreased at higher doses due to residual toxicity in the cells isolated from the treated fetuses. The extent of induced mutation at 200 R was 57 X control. In comparison the ratio of mutants transplacentally induced by a dose of NEU (1 mmole/kg) was 1315 X control.

The spontaneous mutation frequency of somatic cells isolated from a pool of day 13 fetuses from untreated mothers was assembled after 20-25 repeated experiments

that totaled about 600 plates. The mean frequencies for mutation to DT resistance and to 6-thioguanine resistance were 1×10^{-7} and 1.5×10^{-7} , respectively, both on the basis of mutants per surviving cell. This is very low compared to cell lines in vitro and indicates an underlying, very low spontaneous mutation rate. This we estimate to be about 10-50 times less than that reported for cells in culture. One effect of this low mutation frequency is that it enables the detection of low doses of transplacental mutagens, without background noise. The lowest dose of NEU administered transplacentally was 0.5 mmole/kg, which in this direct mutagenicity assay was found to cause an increase in 6-TG mutants that averaged 10X control.

Studies were conducted to systematically investigate the effects of different doses of N-nitrosoethylurea (ENU) and their time of administration (day of gestation) on Syrian golden hamster offspring exposed transplacentally. The most common types of tumor observed in offspring of this species were of peripheral nervous system origin. However, no such tumors (schwannomas) were observed in offspring exposed to ENU before day 10 of gestation. The incidence of such tumors increased progressively from day 10 onwards and the highest incidence of schwannomas was observed in offspring exposed to ENU one day prior to birth. Schwannomas occurred more frequently in female offspring and their incidence was directly proportional to the dose of ENU administered. Previously, we have reported that the sensitivity of the embryo/fetus to the transplacental mutagenic effect of NEU varied greatly during different stages of gestation. For the whole embryo/fetus of the Syrian hamster, this was found to be maximal at day 9. For the whole embryo/fetus of the F344 rat, the greatest sensitivity was found to be at day 11 of gestation. Other studies have attempted to determine the extent of sensitivity of various cell types derived from different organs, when treated with the active alkylating agent NEU 0.2 mmole/kg. In particular, cells isolated from the brain of hamsters treated transplacentally with this dose of NEU were grown under conditions that favor the growth of astrocytes. Maximum mutation induction was found when treatment was early in gestation, the response decreasing as gestation proceeded. The effect of time of treatment on mutation induction in other organs is in progress.

Publications:

None

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05299-05 LCC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Interspecies Differences in Transplacental Carcinogenesis and Tumor Promotion		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: J. M. Ward Chief, Tumor Pathology and Pathogenesis Section LCC NCI		
Others: J. M. Rice Chief LCC NCI L. M. Anderson Expert LCC NCI L. K. Keefer Chief, Chemistry Section LCC NCI A. Hagiwara Guest Researcher LCC NCI		
COOPERATING UNITS (if any) Microbiological Associates, Inc., Bethesda, MD (M. L. Wenk); Program Resources, Inc., Frederick, MD (B. Diwan)		
LAB/BRANCH Laboratory of Comparative Carcinogenesis		
SECTION Tumor Pathology and Pathogenesis Section		
INSTITUTE AND LOCATION NCI, NIH, Frederick, Maryland 21701-1013		
TOTAL MAN-YEARS: 2.2	PROFESSIONAL: 1.6	OTHER: 0.6
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Tumor promotion phenomena in two-stage carcinogenesis were systematically explored in various rodent species in conjunction with transplacental carcinogenesis. Structure-promoting activity relationships of various barbiturates and benzodiazepine tranquilizers were investigated by sequential administration to animals of a transient, low level exposure to a genotoxic carcinogen followed by the test agent under study. Two long-acting hypnotic barbiturates, allobarbitol and aprobarbitol, were found to promote liver carcinogenesis in male rats, while two monosubstituted nonhypnotic barbiturates lacked such activity. Adult inbred strains of mice were found to differ significantly in their susceptibility to the tumor promoting effects of phenobarbital; this susceptibility to tumor promotion appeared to be dominant since the F1 hybrids derived from a cross between the susceptible strain (DBA/2N) and the resistant strain (C57BL/6N) were susceptible to phenobarbital promotion. Unlike the rat and mouse, in Syrian hamsters liver parenchymal cells were resistant to tumor promotion by phenobarbital. Two widely used benzodiazepine tranquilizers, diazepam and oxazepam, were found to promote liver carcinogenesis in mice. Diazepam was more effective than oxazepam and its effect was proportionate to dose. Like a well-known rodent liver tumor promoter, phenobarbital, both diazepam and oxazepam induced hepatomegaly, cytochrome P-450 and cytochrome P-450-dependent aminopyrine N-demethylase activity in mouse hepatocytes.		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI
J. M. Rice	Chief	LCC	NCI
L. M. Anderson	Expert	LCC	NCI
L. K. Keefer	Chief, Chemistry Section	LCC	NCI
A. Hagiwara	Guest Researcher	LCC	NCI

Objectives:

1) To investigate the relationship between molecular structure and promoting activity of various barbiturates and benzodiazepine tranquilizers available commercially for human therapeutics; 2) to determine the cell- and organ-specific tumor promoting effects of barbiturates and benzodiazepines in various rodent species and to explore the genetic and biochemical mechanisms underlying such activities; 3) to characterize and systematically define the limits of organ, strain, and species specificities of promotion by these compounds; and 4) to utilize promotable JB6 mouse epidermal cells, primary rat hepatocytes or bladder or renal tubular epithelial cells to investigate the mechanisms of the promoting action of these agents.

Methods Employed:

In transplacental carcinogenesis studies, precisely timed, pregnant animals of different rodent species are exposed to chemical carcinogens at defined periods during gestation. Offspring from these mothers and young adult animals exposed to carcinogens by conventional routes are subsequently exposed to nongenotoxic agents known or suspected to promote tumorigenesis in one or more organs. Pre-neoplastic proliferative lesions and neoplasms resulting from such treatments are identified and classified by histological, histochemical and ultrastructural parameters. For routine quantitative evaluations of preneoplastic focal proliferative lesions in the liver, an automated image analyzer (Videoplan, Carl Zeiss, Inc., NY) and Zeiss stereology software are used. Commercially unavailable barbiturates and hydantoins of desired molecular structure are synthesized and purified in the Chemistry Section of LCC. For determination of purity of agents used in animal studies, high performance liquid chromatography (HPLC), spectrophotofluorometric methods, and different radioimmunoassays are routinely used. Premalignant JB6 mouse epidermal cells are exposed to suspected tumor promoters and the progression of these cells to tumor cell phenotypes is measured by colony formation in soft agar at 14 days. Ultrastructural changes accompanying promoter-mediated phenotypic transformation are characterized by scanning and transmission electron microscopy.

Major Findings:

Studies were undertaken to test our working hypothesis (JNCI 74: 509, 1985) that only barbiturates that are long-acting hypnotics exhibit tumor promoting activity in rat liver and that, therefore, only 5,5-disubstituted barbiturates can promote tumors. Male F344 rats were given a single initiating dose of N-nitroso-diethylamine (DEN) and beginning 2 weeks later were placed on either normal

diet or diet containing 500 ppm of phenobarbital (PB) or equimolar doses of 5,5-diallylbarbituric acid (ALB), 5-allyl-5-isopropylbarbituric acid (APB), 5-phenylbarbituric acid (MPB), or 5-ethylbarbituric acid (MEB) for the remaining experimental period. Focal hepatocellular proliferative lesions (FHPL), quantified by image analyses/stereology were numerous in rats given PB, ALB, or APB. Incidences of hepatocellular tumors were significantly higher in animals that had received DEN followed by PB, ALB, or APB compared to DEN alone. Neither the incidence of hepatocellular neoplasms nor the number of FHPL was significantly increased by subsequent administration of either 5-monosubstituted (nonhypnotic) barbiturates, MPB or MEB. ALB and APB, like other long-acting hypnotic barbiturates including PB and barbital, promoted liver carcinogenesis in rat liver.

Studies were carried out to investigate the tumor-promoting effects of PB in male Syrian golden hamsters. Potentially preneoplastic hepatocellular hyperplastic foci and hepatocellular neoplasms were studied in weanling hamsters that had received a single initiating dose of either DEN or methylazoxymetranol (MAM) at 5 weeks of age, followed by administration of 500 ppm of PB in drinking water that began 2 weeks after the carcinogen injection and continued to 69 weeks of age. PB failed to promote the development of either preneoplastic hepatocellular foci or hepatocellular neoplasms (adenomas and carcinomas). PB, however, enhanced hepatic cytochrome P-450 activity in hamsters, although no increase in aminopyrine N-demethylase activity was observed in this species.

Adult inbred strains of mice were found to differ significantly in their susceptibility to two-stage liver carcinogenesis initiated by DEN and promoted by PB. Mice of strains DBA/2N and C3H/HeN were susceptible and those of strain C57BL/6N were refractory to liver tumor promotion by PB. The reciprocal F1 progeny between two extreme strains, C57BL/6N and DBA/2N, were also susceptible to liver tumor promotion by PB. Studies on F2 and reciprocal backcross generations are in progress. In addition to hepatocellular carcinogenesis, the male F1 progeny derived from DBA/2N females crossed with C57BL/6N males (D2B6F1) was susceptible to the development of hepatoblastoma when they were exposed to a regimen of DEN initiation and PB promotion. Studies are in progress to 1) clarify the origin and histological characterization of these tumors, 2) determine whether a sex difference exists in susceptibility to the development of hepatoblastomas in D2B6F1 progeny, and 3) detect the expression of transforming gene (oncogene) in these PB-promoted tumors.

Tumor-promoting activity of two widely used benzodiazepine tranquilizers, diazepam and oxazepam, was investigated in male mouse liver. Both these agents promoted development of hepatocellular hyperplastic foci and hepatocellular neoplasms (adenomas and carcinomas) when they were fed in diet to male B6C3F1 mice after initiation by DEN. Diazepam was more effective than oxazepam and its effect was proportionate to dose. Both diazepam and oxazepam induced hepatomegaly, cytochrome P-450 and cytochrome P-450-dependent aminopyrine N-demethylase activity in hepatocytes -- effects similar to those produced by a well-known rodent liver tumor promoter, PB.

Publications:

Diwan, B. A., Palmer, A. E., Ohshima, M. and Rice, J. M.: N-nitroso-N-methyl-urea initiation in multiple tissues for organ-specific tumor promotion in rats by phenobarbital. JNCI 75: 1099-1105, 1985.

Diwan, B. A., Rice, J. M., Ohshima, M. and Ward, J. M.: Interstrain differences in susceptibility to liver carcinogenesis initiated by N-nitrosodiethylamine and its promotion by phenobarbital in C57BL/6NCr, C3H/HeNCr^{MTV}- and DBA/2NCr mice. Carcinogenesis 7: 215-220, 1986.

Diwan, B. A., Rice, J. M. and Ward, J. M.: Tumor-promoting activity of benzo-diazepine tranquilizers, diazepam and oxazepam, in mouse liver. Carcinogenesis 7: 789-794, 1986.

Diwan, B. A., Ward, J. M., Anderson, L. M., Hagiwara, A. and Rice, J. M.: Lack of effect of phenobarbital on hepatocellular carcinogenesis initiated by N-nitrosodiethylamine or methylazoxymethanol acetate in male Syrian golden hamsters. Toxicol. Appl. Pharmacol. (In Press)

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05301-05 LCC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Biology and Pathology of Natural and Experimentally Induced Tumors		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section LCC NCI
Others:	S. Rehm	Visiting Associate LCC NCI
	A. Hagiwara	Guest Researcher LCC NCI
	T. O'Leary	Medical Officer LP NCI
	W. Robey	Research Chemist OD NCI
	P. Nara	Staff Fellow OD NCI
	R. Benveniste	Medical Officer LVC NCI
COOPERATING UNITS (if any) VA Hosp., Pittsburgh, PA (G. Singh); New England Regional Primate Research Center, Southborough, MA (N. King); Washington Regional Primate Research Center, Seattle, WA (C. C. Tsai); Lab. Mol. Microbio., NIAID, Bethesda, MD (E. Santos)		
LAB/BRANCH Laboratory of Comparative Carcinogenesis		
SECTION Tumor Pathology and Pathogenesis Section		
INSTITUTE AND LOCATION NCI, NIH, Frederick, Maryland 21701-1013		
TOTAL MAN-YEARS: 2.5	PROFESSIONAL: 2.5	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.) The pathology and biology of selected experimentally induced and naturally occurring neoplasms of rodents were characterized in order to better elucidate their pathogenesis. Leu-7, a cell surface glycoprotein on human natural killer cells, was found to be an oncofetal antigen of hamster olfactory epithelium, when hamsters were injected with N-nitrosodimethylamine. This antigen, however, was not found in fetuses of other rodents or in their olfactory tumors. So-called "Clara cell" papillary tumors of mouse lung were found to arise from pulmonary alveolar walls and not from Clara cells. Retroviral antigens, including HTLV-III p24 were found in fixed tissue sections from autopsy specimens of humans, monkeys and mice by ABC immunocytochemistry utilizing both monoclonal and polyclonal antibodies. Cross-reactions between STVL-III and HTVL-III were seen. In monkeys, cerebral glia and macrophages contained abundant viral antigens. Retroviral antigens were abundant in early stages of simian and human AIDS but were considerably less prominent in the later stages of these diseases.		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI
S. Rehm	Visiting Associate	LCC	NCI
A. Hagiwara	Guest Researcher	LCC	NCI
T. O'Leary	Medical Officer	LP	NCI
W. Robey	Research Chemist	OD	NCI
P. Nara	Staff Fellow	OD	NCI
R. Benveniste	Medical Officer	LVC	NCI

Objectives:

To study and characterize the biology, pathology and pathogenesis of naturally occurring and experimentally-induced tumors of laboratory animals. To develop animal models for the study of human tumors. To develop methods utilizing experimental animals to better understand the nature, causes and prevention of human cancer.

Methods Employed and Major Findings:

Leu-7, a cell surface glycoprotein with unknown function but previously reported to be found in human natural killer cells, neuroendocrine and neuroepithelial tumors and normal glia of various species, was found to be an oncofetal antigen in the hamster using ABC immunocytochemistry. Normal hamster olfactory epithelium was not shown to contain immunoreactive Leu-7, while fetal and neonatal epithelia were immunoreactive. Dysplastic and neoplastic olfactory epithelium in hamsters injected with N-nitrosodiethylamine were reactive. Neoplastic epithelium of the rat olfactory region was not immunoreactive. Human fetal olfactory epithelium contained individual Leu-7 reactive cells.

In collaboration with investigators at NCI (Timothy O'Leary, W. Gerard Robey, Peter Nara, Raoul Benveniste), New England Regional Primate Center (Norval King) and the Washington Regional Primate Research Center (Che-Chung Tsai), we have localized retroviral antigens (whole virus or gag [core] proteins) to fixed tissue sections of humans, monkeys, and mice with naturally occurring or experimentally induced leukemia, or acquired immune deficiency disease. We have used the ABC immunocytochemical technique and rabbit polyclonal or mouse monoclonal antibodies.

We have successfully identified the viruses in tumor cells and nonneoplastic cells and have shown that the viral titers in fixed tissue sections is dependent on the stage of the immune deficiency disease. The localization of the retroviral proteins in fixed tissue sections should allow pathologists to identify these viruses as potential causes of immune deficiency diseases in humans and animals, including previous necropsies for which no etiologic agent has been found.

A variety of techniques used in experimental pathology including serial sectioning, immunocytochemical localization of pulmonary antigens and ultrastructure have revealed that so called papillary "Clara cell" tumors of mouse lung arise from alveolar walls and not from bronchiolar Clara cells. The papillary tumors had

several features which differed from those of the more typical alveolar type II cell tumors but they also had several features found in normal type II cells. Our studies provide evidence for the alveolar origin of papillary lung tumors of the mouse. The origin of lung tumors in mice has been a subject of great debate during the past few years since an investigator suggested that papillary tumors arose from Clara cells of the bronchioles.

In our initial studies we have demonstrated that the vast majority of papillary lung tumors of mice contain surfactant apoprotein, but never a Clara cell antigen found in normal Clara cells. using rabbit antibodies to rat surfactant apoprotein and Clara cell antigen. More recently Dr. G. Singh of the Pittsburgh VA Hospital has developed for us rabbit antibodies to mouse surfactant apoprotein for use in our immunocytochemical studies. With these antibodies and detailed serial section techniques we have shown that few pulmonary tumors, induced by ENU, in mice arise from bronchioles and the vast majority of papillary tumors arise from alveolar walls and contain lower concentrations of mouse surfactant apoprotein than the usual alveolar type II cell tumors arising from alveolar walls. The ultrastructure of these papillary tumors are difficult to interpret and are potentially confusing for differentiation of Clara cells from alveolar type II cell tumors.

Publications:

Frith, C. H., Pattengale, P. K. and Ward, J. M. (Eds.): A Color Atlas of Hematopoietic Pathology of Mice. Little Rock, Toxicologic Pathology, Inc., 1985, 30 pp.

Gold, L. S., Ward, J. M., Bernstein, L. and Stern, B.: Association between carcinogenic potency and tumor pathology in rodent carcinogenesis bioassays. Fund. Appl. Toxicol. 6: 677-690, 1986.

Reynolds, C. W. and Ward, J. M.: LGL lymphoproliferative diseases in man and experimental animals. In Lotzova, E. and Herberman, R. B. (Eds.): Immunobiology of Natural Killer Cells. West Palm Beach, CRC Press, 1986, Vol. I, pp 193-207.

Reynolds, C. W. and Ward, J. M.: Tissue and organ distribution of NK cells. In Lotzova, E. and Herberman, R. B. (Eds.): Immunobiology of Natural Killer Cells. West Palm Beach, CRC Press, 1986, Vol. I, pp 63-71.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05303-05 LCC												
PERIOD COVERED October 1, 1985 to September 30, 1986														
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Pathogenesis and Promotion of Natural and Induced Tumors														
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 30%;">PI: J. M. Ward</td> <td style="width: 40%;">Chief, Tumor Pathology and Pathogenesis Section</td> <td style="width: 10%;">LCC</td> <td style="width: 10%;">NCI</td> </tr> <tr> <td>Others: S. Rehm</td> <td>Visiting Associate</td> <td>LCC</td> <td>NCI</td> </tr> <tr> <td>A. Hagiwara</td> <td>Guest Researcher</td> <td>LCC</td> <td>NCI</td> </tr> </table>			PI: J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI	Others: S. Rehm	Visiting Associate	LCC	NCI	A. Hagiwara	Guest Researcher	LCC	NCI
PI: J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI											
Others: S. Rehm	Visiting Associate	LCC	NCI											
A. Hagiwara	Guest Researcher	LCC	NCI											
COOPERATING UNITS (if any) Microbiological Associates, Inc., Bethesda, MD (M. Wenk); Program Resources, Inc., Frederick, MD (P. Lynch)														
LAB/BRANCH Laboratory of Comparative Carcinogenesis														
SECTION Tumor Pathology and Pathogenesis Section														
INSTITUTE AND LOCATION NCI, NIH, Frederick, Maryland 21701-1013														
TOTAL MAN-YEARS: 2.0	PROFESSIONAL: 2.0	OTHER:												
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews														
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) The potential mechanisms of action of nongenotoxic tumor promoting and carcinogenic compounds have been studied in rat and mouse liver. Nongenotoxic chemicals may induce tumors in chronic toxicology experiments by enhancing spontaneous carcinogenesis. Two experimental models were developed in aging F344 rats and C3H mice to study the effects of nongenotoxic chemicals on spontaneous hepatocarcinogenesis. All agents studied, including phenobarbital, did not accelerate progression of naturally occurring preneoplastic or neoplastic lesions. Instead phenobarbital appeared either to induce a morphologically distinct population of preneoplastic hepatocellular foci that, in turn, developed into tumors or to promote spontaneously initiated hepatocytes into preneoplastic lesions and tumors. These findings are in marked contrast to those after phenobarbital exposure in initiated rats, whereby the major effect of phenobarbital is to enhance growth of preneoplastic and neoplastic lesions into carcinomas. These studies should help define mechanisms of action of nongenotoxic compounds. In related studies, a model of chronic hepatic hyperplasia is being developed in mice. Levels of DNA synthesis, thymidine kinase and other biochemical parameters are evaluated in relation to hepatic histopathology and organ weights. The chronic hyperplasia induced in liver or kidney may be independent of any carcinogenic effect of a nongenotoxic chemical.														

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI
S. Rehm	Visiting Associate	LCC	NCI
A. Hagiwara	Guest Researcher	LCC	NCI

Objectives:

To characterize the sequential morphologic and biologic steps in the development of cancer after initiation and/or promotion and to study systemically the nature of cellular and organ specificities and species differences in response to tumor promoters.

Methods Employes and Major Findings:

Rodent models were developed to determine if nongenotoxic and genotoxic carcinogens or tumor promoters could promote the development and progression of naturally occurring preneoplastic and neoplastic hepatocellular lesions in rats and mice. In F344 rats, naturally occurring basophilic foci are very common and have been characterized by histochemical methods to lack gamma-glutamyl transferase in contrast to those foci induced by many carcinogens. Nevertheless, some appear to progress spontaneously to adenomas and then carcinomas. In three experiments, we have shown that phenobarbital (PB), sodium nitrite, N-nitrosodiethylamine and di(2-ethylhexyl)phthalate did not increase the size, number or growth rate of these basophilic foci in aging F344 rats. In contrast, phenobarbital induced eosinophilic foci which were GGT-positive and progressed to adenoma more quickly than the basophilic foci in rats exposed to PB. We have developed a similar model in aging C3H mice. Mice one year of age were exposed to PB for up to 36 weeks. The number of eosinophilic foci per liver was greatly increased by 12 weeks and the number of adenomas by 24 weeks. No effect was seen on naturally occurring basophilic preneoplastic and neoplastic lesions nor was progression to carcinoma or mortality affected. Our mouse and rat experiments provide evidence that a major effect of PB on rodent liver is the induction of eosinophilic proliferative lesions which progress to neoplasms rather than from enhancement of the previously existing preneoplastic or neoplastic lesions. We suggest that PB in these systems have an effect on spontaneously initiated cells or noninitiated but PB-susceptible cells.

We are developing a system to study the role of hyperplasia on tumor promotion and carcinogenesis in mouse liver by nongenotoxic chemicals. We have fed diets of water containing PB, di(2-ethylhexyl)phthalate, acetaminophen, or barbitol to mice for periods of from 2-24 weeks. Parameters of hyperplasia to be measured include liver weight, hepatopathology, DNA synthesis (as measured by tritiated thymidine uptake), total liver DNA, thymidine kinase, and metallothionein levels. We have previously shown that acetaminophen can produce chronic hepatotoxicity without evidence of carcinogenesis. With our new model systems we are attempting to confirm that chronic toxicity and organ enlargement are indicative of chronic hyperplasia, a biological state suggested as a prerequisite for the carcinogenic effects of nongenotoxic chemicals. This applies both to liver and to thyroid follicular epithelium which is effectively promoted by goitrogenic stimuli.

Publications:

Ohshima, M. and Ward, J. M.: Dietary iodine deficiency as a tumor promoter and carcinogen in F344/NCr male rats. Cancer Res. 46: 877-883, 1986.

Ward, J. M., Diwan, B. A., Ohshima, M., Hu, H., Schuller, H. M. and Rice, J. M.: Tumor initiating and promoting activities of di(2-ethylhexyl)phthalate in vivo and in vitro. Environ. Health Perspect. 65: 279-291, 1986.

Ward, J. M. and Ohshima, M.: Evidence for lack of promotion of the growth of the common naturally occurring basophilic focal hepatocellular proliferative lesions in aged F344/NCr rats by phenobarbital. Carcinogenesis 6: 1255-1259, 1985.

Patents:

None

CONTRACT IN SUPPORT OF PROJECT NUMBERS:

Z01CP04582-11 LCC
Z01CP05157-07 LCC
Z01CP05093-08 LCC
Z01CP05299-05 LCC
Z01CP05303-05 LCC
Z01CP05399-03 LCC

MICROBIOLOGICAL ASSOCIATES (N01-CO-41014)

Title: Non-SPF Rodent Holding Facility for the Laboratory of Comparative Carcinogenesis

Current Annual Level: \$342,834

Man Years: 4

Objectives: The purpose of this contract is to provide support services for the Laboratory of Comparative Carcinogenesis for long-term holding, treatment and observation of rodents in carcinogenesis investigations emphasizing lifetime tumor induction in rodents and related activities. The contract is specifically utilized for conducting experiments that require species or strains of rodents not available from the Frederick Cancer Research Facility (FCRF) animal production area, since such animals cannot be introduced into the LCC animal research facilities at the FCRF. The contract provides holding facilities for 1,000 rats, 2,000 mice, and 500 hamsters or their equivalent.

Protocols are developed in collaboration with LCC investigators and approved by an LCC project officer. Protocols involve the preparation, handling and administration of chemical solutions to animals according to NCI guidelines for the safety of personnel; specifications for holding, treatment, and data collection (including gross pathology data) for mice, hamsters, rats and related species; administration of chemical carcinogens to animals by skin painting, gavage, parenteral injection or other routes; storage of labile animal diets, reagents, tissues or other materials under controlled temperature conditions; qualitative or quantitative analysis of carcinogen preparations or of tissues of carcinogen-treated animals and other necessary details are provided by LCC investigators. All fixed tissue specimens from carcinogenesis studies are sent to FCRF for histology and evaluation by NCI pathologists.

Major Contributions: This contract has made possible research involving administration of chemical carcinogens to laboratory animals that could not have been accomplished at FCRF, including studies on the effects of inorganic carcinogens and tumor promoters in different rat strains, ENU in congenic strains of mice varying in their expression of murine retroviruses (in collaboration with Dr. Janet Hartley, NIAID), and carcinogenesis in Mongolian gerbils, which vary strikingly from other rodents in their response to chemical carcinogens.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05352-04 LCC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Metabolic and Pharmacological Determinants in Perinatal Carcinogenesis		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: L. M. Anderson Expert		LCC NCI
Others: J. M. Rice Chief, Perinatal Carcinogenesis Section		LCC NCI
J. M. Ward Chief, Tumor Pathology and Pathogenesis Section		LCC NCI
A. Hagiwara Guest Researcher		LCC NCI
COOPERATING UNITS (if any) Program Resources, Inc., Frederick, MD (H. Issaq); American Health Foundation, Valhalla, NY (S. Hecht)		
LAB/BRANCH Laboratory of Comparative Carcinogenesis		
SECTION Perinatal Carcinogenesis Section		
INSTITUTE AND LOCATION NCI, NIH, Frederick, Maryland 21701-1013		
TOTAL MAN-YEARS: 1.25	PROFESSIONAL: 0.5	OTHER: 0.75
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> This project addresses carcinogenesis during the perinatal period with regard both to mechanisms underlying susceptibility and to assessment of public health related phenomena. In a pharmacogenetic investigation of transplacental carcinogenesis by methylcholanthrene in mice, genetic backcrosses of C57BL/6 and DBA/2 mice were employed to produce fetuses which were, in the same mother, either inducible or noninducible for the enzymes which metabolize this carcinogen. When methylcholanthrene treatment was preceded by exposure of the mother to the noncarcinogenic enzyme inducer, beta-naphthoflavone, the numbers of tumors in both lung and liver were reduced, but only in induction-responsive fetuses. This phenomenon was observed in fetuses of both induction-responsive and nonresponsive mothers, even though progeny of DBA mothers developed more tumors than did those of (C57BL/6 X DBA)F1 mothers given the same dose of methylcholanthrene. Furthermore, exposure to these agents had long-term complex effects on the responsiveness of the progeny to enzyme induction later in life. In other experiments in progress involving nitrosamines, N-nitrosodimethylamine and N-nitrosodiethylamine are being examined for their ability to cause neurogenic tumors in mice by transplacental exposure; and a tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane (NNK), is being studied for transplacental tumorigenic potential. Both of these efforts may yield results of direct relevance to the etiology of certain childhood cancers. Another study of potential public health importance is assessing polychlorinated biphenyls as promoters and enhancers of tumors initiated by nitrosamine in the infant mouse, with examination of age at treatment, action of specific PCB congeners, and long-term biochemical changes in the tissues. </p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

L. M. Anderson	Expert	LCC	NCI
J. M. Rice	Chief, Perinatal Carcinogenesis Section	LCC	NCI
J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI
A. Hagiwara	Guest Researcher	LCC	NCI

Objectives:

To identify and characterize the cellular and organismal factors that determine the susceptibility of immature animals to carcinogens and that influence the development of tumors initiated during the perinatal period; to assess the transplacental effects of important environmental carcinogens; to develop animal models for study of the etiology of common human childhood cancers.

Methods Employed and Major Findings:

Genetic Determinants of Susceptibility in a Pharmacogenetic Model: The role of metabolic activation/detoxification of chemical carcinogens as a determinant of susceptibility to carcinogenesis has been studied in a pharmacogenetic mouse model. Appropriate genetic crosses were made between a mouse strain, C57BL/6, which is responsive to induction of metabolism of polycyclic aromatic hydrocarbon carcinogens, and DBA, which is not, to generate both responsive and nonresponsive fetuses in mothers which are either responsive or nonresponsive. In a recently completed experiment it was shown that both maternal and fetal genotype influenced the numbers of tumors caused by transplacental methylcholanthrene (MC): responsive fetuses got more liver and lung tumors than nonresponsive littermates, but nonresponsive mothers permitted greater transplacental effectiveness of injected MC. In a new experiment currently in progress, the transplacental exposure to MC (5-100 mg/kg in olive oil, i.p.) has been preceded by treatment of the mothers with β -naphthoflavone (β NF) (150 mg/kg), a noncarcinogenic enzyme inducer. Metabolite phenotyping has been carried out at 13 months of age by application of an assay for ^{14}C -MC metabolism 48 hours after induction with MC (80 mg/kg). Lung and liver tumors were counted and evaluated histologically. Preliminary results indicate that prior treatment with the inducer reduced the number of tumors initiated in both lungs and livers of fetuses of both sexes, but only if the fetus was of inducible phenotype. This is the first direct evidence that fetuses may be protected against a carcinogen by induction of detoxication enzymes. Furthermore, analysis of the degree of MC metabolism by liver homogenates from the offspring reveals that exposure of the fetuses to MC and β NF appears to have had permanent effects on ability to respond to enzyme induction later in life -- an imprinting action. This experiment will be carried to completion and biochemical and pharmacokinetic analysis of fetal and maternal metabolic contributions carried out.

Transplacental Carcinogenesis by Nitroso Compounds in C3H Mice: Neurogenic tumors are known to be caused transplacentally by nitrosoureas in C3H mice, and we have shown that mouse fetuses, in contrast to rat fetuses, have the ability to metabolize nitrosamines and thus, presumably, to activate them to proximate carcinogens. In an ongoing experiment fetuses of C3H mice have been exposed to N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), or to N-nitrosoethylurea (NEU) as a positive

control. Pregnant mothers were treated with the chemicals (0.5 mmole/kg) on gestation day 16 or 19. Tumors have been evaluated in mice killed when moribund. Some neurogenic tumors have appeared in the latter group, and liver and lung tumors have been common in all groups, confirming the transplacental potency of the nitrosamines. Analysis of some of these tumors for oncogenes is planned in collaboration with the Developmental Biology Working Group.

Transplacental Effectiveness of a Tobacco-Specific Nitrosamine: Tobacco is a major source of exposure of fetuses and children to nitrosamines, especially of several tobacco-specific nitrosamines that are present in relatively large amounts. The most potent of these as a carcinogen in adult animals, is 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). This compound is known to be metabolized (activated) in fetal mouse tissues, but its effectiveness as a transplacental carcinogen has not been studied. In collaboration with Dr. S. Hecht at the American Health Foundation, we have exposed fetuses of C3H mothers, fathered by C57BL/6 males, to a high dose of NNK (100 mg/kg) on days 14, 16, and 18 of gestation by i.p. treatment. This treatment is to be followed in some cases by postnatal treatment with tumor promoters, phenobarbital or polychlorinated biphenyls. The progeny will be assessed for tumors of the lung, liver, nasal cavity, lymphoid system, kidney, thyroid, and other organs.

PCB Promotion and Suppression of Tumors Initiated in the Neonatal Mouse: A single dose of polychlorinated biphenyls (PCBs) given to infant mice 4 days after initiation of lung and liver tumors by NDMA resulted in increased numbers of lung tumors and reduced numbers but increased size of liver tumors. These changes were associated with retention in the bodies of the mice of two hexachlorobiphenyl congeners. In the current phase of this project we are examining these effects as related to age at treatment, the actions of the retained congeners given singly and in combination, and long-term biochemical changes in the tissues affected by the PCBs as related to tumorigenesis. Tumors have been initiated by treatment of mice at 4 days with 5 mg/kg NDMA or at 6 weeks with 10 mg/kg. This treatment has then been followed by the PCBs mixture, Aroclor 1254, or one or both of 2 congeners, 2,4,5,2',4',5'- or 2,3,4,2',4',5'-hexachlorobiphenyl. Some mice will be killed at 16 weeks in evaluation of effects on lung tumors, whereas others will be maintained for longer period to investigate long-term biochemical and histological activity.

Publications:

Anderson, L. M., Jones, A. B., Riggs, C. W., and Ohshima, M.: Fetal mouse susceptibility to transplacental lung and liver carcinogenesis by 3-methylcholanthrene: Positive correlation with responsiveness to inducers of aromatic hydrocarbon metabolism. Carcinogenesis 6: 1389-1393, 1985.

Anderson, L. M., Ward, J. M., Fox, S., and Riggs, C. W.: Effects of a single dose of polychlorinated biphenyls to infant mice on N-nitrosodimethylamine-initiated lung and liver tumors. Int. J. Cancer (In Press)

Rice, J. M., Donovan, P. J., and Anderson, L. M.: Mutagenesis and carcinogenesis. In Fabro, S. and Scialli, A. R. (Eds.): Drug and Chemical Action in Pregnancy. New York, Marcel Dekker (In Press)

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05353-04 LCC															
PERIOD COVERED October 1, 1985 to September 30, 1986																	
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Sensitivity Factors in Special Carcinogenesis Models																	
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 60%;">L. M. Anderson Expert</td> <td style="width: 25%; text-align: right;">LCC NCI</td> </tr> <tr> <td colspan="3" style="padding-top: 10px;">Others:</td> </tr> <tr> <td style="width: 15%;">J. M. Rice</td> <td style="width: 60%;">Chief, Perinatal Carcinogenesis Section</td> <td style="width: 25%; text-align: right;">LCC NCI</td> </tr> <tr> <td>J. M. Ward</td> <td>Chief, Tumor Pathology & Pathogenesis Section</td> <td style="text-align: right;">LCC NCI</td> </tr> <tr> <td>A. Hagiwara</td> <td>Guest Researcher</td> <td style="text-align: right;">LCC NCI</td> </tr> </table>			PI:	L. M. Anderson Expert	LCC NCI	Others:			J. M. Rice	Chief, Perinatal Carcinogenesis Section	LCC NCI	J. M. Ward	Chief, Tumor Pathology & Pathogenesis Section	LCC NCI	A. Hagiwara	Guest Researcher	LCC NCI
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J. M. Ward	Chief, Tumor Pathology & Pathogenesis Section	LCC NCI															
A. Hagiwara	Guest Researcher	LCC NCI															
COOPERATING UNITS (if any) Temple University, Philadelphia, PA (G. Harrington and P. Magee) University of South Florida, Tampa, FL (A. Giner-Sorolla)																	
LAB/BRANCH Laboratory of Comparative Carcinogenesis																	
SECTION Perinatal Carcinogenesis Section																	
INSTITUTE AND LOCATION NCI, NIH, Frederick, Maryland 21701-1013																	
TOTAL MAN-YEARS: <div style="text-align: right;">1.25</div>	PROFESSIONAL: <div style="text-align: right;">1.0</div>	OTHER: <div style="text-align: right;">0.25</div>															
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) Human subjects</td> <td><input type="checkbox"/> (b) Human tissues</td> <td><input checked="" type="checkbox"/> (c) Neither</td> </tr> <tr> <td><input type="checkbox"/> (a1) Minors</td> <td colspan="2"></td> </tr> <tr> <td><input type="checkbox"/> (a2) Interviews</td> <td colspan="2"></td> </tr> </table>			<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input checked="" type="checkbox"/> (c) Neither	<input type="checkbox"/> (a1) Minors			<input type="checkbox"/> (a2) Interviews								
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<input type="checkbox"/> (a2) Interviews																	
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Factors involved in modulation effects of chemical carcinogens have been studied in several murine models. (1) Co-administration of ethanol has been found to have pronounced effects on the metabolism, distribution and toxic effects of the environmental carcinogen, N-nitrosodimethylamine (NDMA), including doses of NDMA giving blood levels comparable to those measured in many humans and causing, on the other hand, an increased incidence of lung tumors in mice. Ethanol given simultaneously with NDMA reduced the hepatotoxicity of the chemical, but caused a large increase in circulating levels of NDMA, presumably through competitive inhibition of NDMA metabolism. NDMA demethylase was not induced at the concentrations of ethanol used. A dose of 550 ppb NDMA resulted in an average blood steady-state level of 0.9 ppb, and also caused a significant doubling in incidence of lung tumors in strain A mice. This model system thus has considerable potential for mechanistic studies related to the metabolic epidemiology of cancer. (2) The induction of metabolism of NDMA in various mouse strains by acetone, pyrazole, and isopropanol is being systematically investigated with the intent to develop in vivo pharmacogenetic-pharmacokinetic models. Work is continuing on protection against polycyclic aromatic hydrocarbon carcinogenesis by enzyme induction. (3) Effects of N-nitrosocimetidine on tumor development are being studied with both skin and mammary tumor models, with several experiments nearing completion. (4) A project on tumorigenesis in athymic nude mouse skin, showing high incidence of squamous tumors in this model, has been completed and terminated.</p>																	

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

L. M. Anderson	Expert	LCC	NCI
J. M. Rice	Chief, Perinatal Carcinogenesis Section	LCC	NCI
J. M. Ward	Chief, Tumor Pathology & Pathogenesis Sec.	LCC	NCI
A. Hagiwara	Guest Researcher	LCC	NCI

Objectives:

To investigate mechanisms for differences in effects of chemical carcinogens as a function of chemical structure and of inherent genetic differences among species, strains, and target organs. The project utilizes agents of unique chemical or human-exposure interest and special animal models where susceptibility characteristics present favorable situations for mechanistic analysis.

Methods Employed and Major Findings:

N-Nitrosodimethylamine (NDMA)-Ethanol Interactions: The metabolism, distribution, and toxic effects of the environmental carcinogen NDMA have been investigated in mice in relation to interactions with co-administered ethanol. NDMA was administered in the drinking water of Swiss mice at doses of 0.5-50 ppm with or without 10-30% ethanol. Levels of NDMA were measured in blood, liver, kidney, lung, and brain by methylene chloride extraction followed by thermal energy analysis. Toxic effects in the livers were evaluated histopathologically. Enzymatic activity toward NDMA in liver was measured in vitro using the postmitochondrial supernatant fraction and a colorimetric assay for formaldehyde. Ethanol did not induce but rather competitively inhibited metabolism of NDMA in the liver, thereby reducing toxic effects at high doses and causing increased levels of the carcinogen in blood and peripheral targets at all doses (0.5 - 50 ppm). At a dose of 0.5 ppb NDMA was detected in about half of the samples taken at levels of 0.5-2 ppb. Similar levels are common in human blood. This same dose of 0.5 ppb was found to cause an increase in lung tumors in strain A mice. A study of the effects of coadministration of ethanol on lung tumor incidence is in progress. These results may provide important insights into the factors contributing to and the cancer risk implied by human blood burdens of NDMA.

Protective Effects of Induction of Carcinogen Metabolism: A study has been completed showing that β -naphthoflavone, a noncarcinogenic inducer of mixed function oxygenase and various detoxification enzymes acting on polycyclic aromatic hydrocarbon carcinogens, provided a high degree of protection against the action of methylcholanthrene, but only in those mouse strains genetically responsive to induction. For this study, the β NF (150 mg/kg) was given 24 hrs before a carcinogenic dose of MC (20 mg/kg) weekly for 12 weeks. The mouse strains used were C57BL/6, BALB/c, C3H, Swiss, DBA/2, and AKR. The mice were killed after 1 year and evaluated for tumors. Further experiments of this type are being planned with other inducers and carcinogens and other genetic models, including a congenic mouse strain with the gene for responsiveness (Ah) included in the genome of a nonresponsive strain. Also, biochemical work is in progress to enable similar experiments with nitrosamines; mouse strains are being surveyed for inducibility of NDMA demethylase by agents such as isopropanol and pyrazole. NDMA demethylase is being measured using the

postmitochondrial supernatant fraction of liver and a colorimetric assay for formaldehyde. Extension of the principle to the human will be attempted using primary and cultured hair follicles.

Biological Activity of N-Nitrosocimetidine (NCM): NCM is a compound of both public health and theoretical chemical interest. It is a derivative of a commonly-used drug and, in spite of a demonstrated capability for genotoxic damage, it has proved to be noncarcinogenic in several biological tests. We have completed and published a study of effects the of chronically-administered NCM given throughout the life span of mice, showing no pronounced tumorigenic effects but some evidence that NCM could act on tumors once formed. Several tests of this possibility are currently in progress, utilizing skin tumors in Sencar mice and mammary tumors in B6C3F1 mice. In the skin study, female Sencar mice were treated topically with 1 mg NCM twice weekly for 4 weeks, followed by twice weekly applications of TPA (1.0 µg). They are currently being observed for tumors. In the mammary tumor study, female (C57BL/6 x DBA/2) F₁ mice were given 4 weekly intragastric doses of 1 mg DMBA to initiate mammary tumors. This treatment was followed by NCM (1.13 g/liter drinking water), with controls receiving water only. The mice are being killed when moribund and evaluated for tumors. Preliminary results indicate that NCM may have effectiveness as a tumor initiator, as would be predicted from its chemical structure.

Skin Carcinogenesis in Nude Mice: A study has been completed demonstrating high susceptibility of nude mouse skin to carcinogenesis by UV light and chemical carcinogens, establishing this athymic model as a new system for study of determining factors in skin carcinogenesis. The chemicals included 7,12-dimethylbenz[a]anthracene and N-nitrosoethylurea applied topically, and in some cases followed by TPA. UV irradiation took place twice weekly. The mice were killed when moribund or after 13 months and all skin tumors evaluated histopathologically. Also, sebaceous adenomas were discovered to be a major category of tumor caused by a single high dose of 7,12-dimethylbenz[a]-anthracene and these were often associated with hyperplasia of the overlying epidermis. This also may be a useful model for investigation of autocrine factors in tumor promotion. No further experiments are planned.

Publications:

Anderson, L. M., Giner-Sorolla, A., Haller, I. M. and Budinger, J. M.: Effects of cimetidine, nitrite, cimetidine plus nitrite, and nitrosocimetidine on tumors in mice following transplacental plus chronic lifetime exposure. Cancer Res. 45: 3561-3566, 1985.

Anderson, L. M. and Rice, J. M.: Sensitivity of the skin of athymic nude mice to causation of tumor by chemical carcinogens or ultraviolet light. JNCI (In Press)

Anderson, L. M. and Saraswathy, S.: Protection against tumorigenesis by 3-methylcholanthrene in mice by β-naphthoflavone as a function of inducibility of methylcholanthrene metabolism. Cancer Res. 45: 6384-6389, 1985.

Pylypiw, H. W., Zimmerman, F., Harrington, G. W. and Anderson, L. M.: Apparatus for trace analysis of volatile N-nitrosamines in small samples. Anal. Chem. 52: 2996-2997, 1985.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05399-03 LCC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Oncogene Expression in Chemically Induced Tumors		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal investigator.) (Name, title, laboratory, and institute affiliation) PI: J. M. Rice Chief LCC NCI Others: A. O. Perantoni Microbiologist LCC NCI M. Watatani Visiting Fellow LCC NCI C. D. Reed Senior Health Services Officer LCC NCI J. M. Ward Chief, Tumor Pathology & Pathogenesis Section LCC NCI		
COOPERATING UNITS (if any) Microbiological Associates, Inc., Bethesda, MD (M. L. Wenk)		
LAB/BRANCH Laboratory of Comparative Carcinogenesis		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Frederick, Maryland 21701-1013		
TOTAL MAN-YEARS: 3.0	PROFESSIONAL: 2.5	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) The expression of activated cellular oncogenes in chemically induced rat tumors and the relationship of oncogene expression to progression from the normal to the neoplastic phenotype are studied using 3T3 transfection and hybridization techniques and monoclonal antibodies directed against the specific oncogene products. Five types of tumors have been generated by single injection of F344 rats using various alkylating agents: renal mesenchymal tumors induced by methyl(methoxymethyl)-nitrosamine (DMN-OMe), intestinal adenocarcinomas induced by methyl(acetoxymethyl)-nitrosamine (DMN-OAc), hepatocellular carcinomas induced by intraportal injection of DMN-OAc followed by phenobarbital promotion, and gliomas and schwannomas induced by transplacental exposure to nitrosoethylurea (ENU). DNA purified from these tumors is utilized for 3T3 transfection assays and in Southern blot hybridizations with available oncogene probes. Tissue specific activated oncogene sequences of rat origin were found in two of these model systems. Rat renal mesenchymal tumors frequently contained K-ras (15/33); only 1/33 contained a different activated gene, and that was N-ras, a member of the same family. Schwannomas commonly contained an activated neu (erbB-2) gene (6/13); one such tumor contained both neu and N-ras. The neu gene is totally unrelated to the ras family and this finding diminishes the likelihood that selective isolation of activated oncogenes in chemically induced tumors is a technical artifact. No comparable frequency of activated genes was found in 59 chemically induced gliomas or in more than 180 hepatocellular neoplasms, which sporadically revealed N-ras (1 glioma) or K- or H-ras (six liver tumors). Oncogene proteins were localized to fixed tissue sections of tumors and nonneoplastic lesions by avidin-biotin immunocytochemistry. With monoclonal or polyclonal antibodies to the proteins or their peptides, H-ras p21 could be identified in Harvey sarcoma virus-induced sarcomas and splenic erythroblasts but not in a variety of naturally occurring and induced tumors, even those proven to have activated H-ras oncogenes.		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. M. Rice	Chief	LCC	NCI
A. O. Perantoni	Microbiologist	LCC	NCI
M. Watatani	Visiting Fellow	LCC	NCI
J. M. Ward	Chief, Tumor Pathology & Pathogenesis Section	LCC	NCI
C. D. Reed	Senior Health Services Officer	LCC	NCI

Objectives:

To identify activated oncogene sequences in specific types of chemically induced neoplasms in rats in comparison with normal, nonneoplastic tissues in the same animals. To isolate and characterize the oncogene sequences found as mutant or wild-type alleles. To generate immunologic probes for available oncogene products and to apply these to detect expression of oncogenes at different stages of tumor development as well as during embryonic or fetal development.

Methods Employed:

Tumor generation: Five particular types of neoplasms in F344 rats are being induced to supply DNA for transfection experiments. These tumors include renal mesenchymal tumors induced by single neonatal i.p. injections of methyl(methoxymethyl)nitrosamine (DMN-OMe); intestinal adenocarcinomas induced by single i.p. injections of methyl-(acetoxymethyl)nitrosamine (DMN-OAc) in 5 week-old animals; hepatocellular carcinomas induced by single intraportal injections of DMN-OAc to 100-gram rats which were subjected to partial hepatectomies 21-24 hours previously and subsequently promoted with phenobarbital in the diet; and gliomas of the brain and schwannomas of cranial, spinal, and peripheral nerves induced by single transplacental exposure to nitrosoethylurea (ENU) at 15 days gestation. Each of these alkylating agents has a short half-life. Predictable latency periods after the single instillation until the development of the desired tumor and documented histogenetic sequences are associated with the development of each of these experimental tumor types.

Transfection experiments: DNA is purified from the various tumors and appropriate control tissues by standard protocols, precipitated with calcium phosphate, and incubated with a clone of NIH 3T3 cells known to be readily transfectable. Oncogene-positive clones are isolated to provide a source of DNA for oncogene sequence analysis.

Oncogene screening: Available clonal oncogenes are radiolabeled and hybridized with tumor DNAs that have been digested with an appropriate restriction enzyme, separated by gel electrophoresis, and transferred to nitrocellulose. Rat oligonucleotide sequences and specific oncogene sequences are identified by blotting techniques. Activated oncogenes will be sequenced to determine their similarity to the wild-type allele.

Oncogene expression: Depending upon oncogene product availability, monoclonal antibodies will be developed specifically for that product and will be used in immunohistochemical procedures to characterize expression of the oncogene during different stages of histogenesis in these chemically induced tumors.

Major Findings:

Methyl(methoxymethyl)nitrosamine induced predominantly renal mesenchymal tumors in F344 rats following neonatal administration. In addition, we observed renal epithelial tumors, hepatocellular proliferative lesions (both preneoplastic and neoplastic), nasal cavity adenocarcinoma, peripheral lung adenoma, bronchogenic adenocarcinoma, and pituitary adenomas. When calcium-phosphate precipitated DNA from primary tumors was applied to cultures of NIH 3T3 cells, preparations from 15 of 33 renal mesenchymal tumors, 1 hepatocellular carcinoma, 1 nasal cavity tumor, and 1 lung adenocarcinoma contained transforming activity. Transformants derived in this protocol all contained rat-specific sequences and an activated ras gene family member. With the exception of one transformant from renal mesenchymal tumor DNA that contained rat-specific N-ras sequences, all other transformants tested exhibited K-ras sequences, including those derived from epithelial tumors of extrarenal origin.

Transplacental exposure of F344 rats to ethylnitrosourea at 16 days gestation induced neurogenic tumors almost exclusively. Of 59 anaplastic gliomas, 1 contained transforming activity while 6 of 13 DNA preparations from schwannomas transformed cultures of NIH 3T3 cells. Southern blot analysis of these transformants identified the presence of a new family of activated sequences in primary tumors. Transformants from the schwannomas exhibited rat-specific erbB-2 or neu sequences; the single transformant from the glial DNA had N-ras sequences.

Study of activation of oncogenes in hepatocellular lesions initiated with methyl (acetoxymethyl)nitrosamine and promoted with phenobarbital resulted in the identification of five hepatocellular tumors that contained transforming sequences. Of 183 liver lesions of various types observed in 64 animals, preparations from 4 carcinomas induced transformation of NIH 3T3 cells; of these, 3 showed activated K-ras sequences, while the fourth had H-ras sequences. A fifth transformant was derived from a hepatocellular adenoma and is yet to be characterized, although it contains no rat-specific ras sequences.

Selective activation of ras sequences in renal tumors, erbB-2 (neu) sequences in schwannomas, and no specific pattern in gliomas or hepatocellular tumors is difficult to reconcile with any hypothesis of artifactual "activation" during the DNA isolation/transfection process and strengthens the argument for a role of these genes in carcinogenesis in certain tissues.

Oncogene proteins have been localized in fixed tissue sections to nonneoplastic cells using monoclonal and polyclonal antibodies to oncogene proteins or synthetic peptide fragments in the avidin biotin immunocytochemical (ABC) technique. The most successful antibodies used have been a rat monoclonal antibody to H-ras p21 (Furth et al., J. Virol. 43: 294-304, 1982), a sheep polyclonal IgG to a synthetic peptide homologous to a 20 amino acid peptide in the carboxyterminal end of H-ras p21 (Ward et al., Carcinogenesis 7: 645-651, 1986) and most recently mouse monoclonal antibodies to ras p21 proteins produced by transformed tumor cells (Santos, E., unpublished observations). Each antibody has its own specificity and immunomorphologic appearance in tumor or nonneoplastic cells. We believe our paper in

Carcinogenesis to represent the first published report demonstrating membrane staining of the membrane antigen H-ras p21 in fixed tissues routinely used in pathology. We have also shown, for the first time, that immunoreactivity can be abolished or greatly reduced by absorption of the specific IgG with the antigen. The techniques we have developed and refined should allow investigators to localize the transforming gene protein products to specific cell types and to allow clearer understanding of the role of these gene products in carcinogenesis.

Publications:

Gastl, G., Ward, J. M. and Rapp, U. R.: Immunocytochemistry of oncogenes. In Polak, J. M. (Ed.): Immunocytochemistry-Practical Applications in Pathology and Biology. Bristol, England, Wright-PSG (In Press)

Sukumar, S., Perantoni, A., Reed, C., Rice, J. M. and Wenk, M. L.: Activated K-ras and N-ras oncogenes in primary renal mesenchymal tumors induced in F344 rats by methyl(methoxymethyl)nitrosamine. Mol. Cell. Biol. (In Press)

Ward, J. M., Pardue, R. L., Junker, J. L., Takahashi, K., Shih, T. Y. and Weislow, O. S.: Immunocytochemical localization of Ras-Ha p21 in normal and neoplastic cells in fixed tissue sections from Harvey sarcoma virus-infected mice. Carcinogenesis 7: 645-651, 1986.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05465-02 LCC																									
PERIOD COVERED October 1, 1985 to September 30, 1986																											
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) The Regulatory Role of Retinoids and Growth Factors in Tissue Differentiation																											
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 40%;">U. I. Heine</td> <td style="width: 40%;">Chief, Ultrastructural Studies Section</td> <td style="width: 10%;">LCC</td> <td style="width: 10%;">NCI</td> </tr> <tr> <td colspan="5" style="padding-top: 10px;">Others:</td> </tr> <tr> <td></td> <td>J. L. Junker</td> <td>Staff Fellow</td> <td>LCC</td> <td>NCI</td> </tr> <tr> <td></td> <td>A. B. Roberts</td> <td>Research Chemist</td> <td>LC</td> <td>NCI</td> </tr> <tr> <td></td> <td>M. B. Sporn</td> <td>Chief</td> <td>LC</td> <td>NCI</td> </tr> </table>			PI:	U. I. Heine	Chief, Ultrastructural Studies Section	LCC	NCI	Others:						J. L. Junker	Staff Fellow	LCC	NCI		A. B. Roberts	Research Chemist	LC	NCI		M. B. Sporn	Chief	LC	NCI
PI:	U. I. Heine	Chief, Ultrastructural Studies Section	LCC	NCI																							
Others:																											
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	A. B. Roberts	Research Chemist	LC	NCI																							
	M. B. Sporn	Chief	LC	NCI																							
COOPERATING UNITS (if any) Biological Products Laboratory, Program Resources, Inc., FCRF, Frederick, MD (E. F. Munoz)																											
LAB/BRANCH Laboratory of Comparative Carcinogenesis																											
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SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> An experimental model has been established to study the regulatory role of retinoids in growth and differentiation. Retinoid deprivation during embryonal development of the chick and quail causes abnormalities in organs of epithelial and mesenchymal origin, most dramatically preventing the formation of the extra-embryonal circulatory system. By light microscopy, TEM and SEM, retinoid-deficient quail embryos (5 somite stage to 2 days of age) show the following sequence of events leading to vascular abnormalities: 1) a cardia bifida forms if development of the heart is retarded before fusion of the primitive endothelial heart tubes takes place; 2) a single enlarged ventricle develops in situ inverso if retardation manifests itself at a later stage; and 3) in all cases, the omphalomesenteric veins that normally extend caudal from the sinus venosus are not developed and the endocardium of the heart terminates as a blind pouch in the mid-ventral region of the animal. The results thus provide evidence for the failure to establish, at the level of the omphalomesenteric veins, a connection between the embryonal and extraembryonal circulatory system in the retinoid-deficient quail embryos. Since the extracellular matrix is of importance in normal cell growth, cell movement and differentiation, and thus may be a decisive factor in coordinated differentiation of the heart in the early embryo, we are testing the role of matrix proteins such, as fibronectin and laminin, in early development using our model system of the retinoid-deficient quail embryo. These studies are now extended to evaluate the role of growth factors, especially TGF-β, in cell growth and differentiation. </p>																											

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Ursula I. Heine	Chief, Ultrastructural Studies Section	LCC	NCI
James L. Junker	Staff Fellow	LCC	NCI
Anita B. Roberts	Research Chemist	LC	NCI
Michael B. Sporn	Chief	LC	NCI

Objectives:

To investigate specific developmental pathways in retinoid-deficient quail embryos in order to further our understanding of the molecular mechanisms of retinoid action in modifying cell differentiation and regulating cell proliferation, and to investigate the role of the transforming growth factor TGF- β in cell differentiation, inflammation and tissue repair.

Methods Employed and Major Findings:

We have developed a model system that may have valuable applications in the study of molecular mechanisms, whereby retinoids control differentiation and proliferation of cells of epidermal and mesenchymal origin. In vitro studies demonstrate that retinoid deprivation produces defects in the development of the circulatory system by culture of normal 24-hr embryos on retinoid-deficient agar medium; however, normal development is observed upon culture of retinoid-deficient embryos on retinoid-containing agar medium. Extension of this study to the quail embryo indicates that in vivo retinoid deprivation causes abnormalities in organs of epithelial and mesenchymal origin, most dramatically preventing the formation of the extraembryonal circulatory system in the avian embryo. Our in vivo studies, using quail embryos between the 5-15 somite stage, as well as 2 and 2.5-day-old embryos, show that the basis for the latter defect is the failure of the primitive heart tubes to open at their posterior end, thus preventing the formation of omphalomesenteric veins normally connecting the embryonal with the extraembryonal circulatory system. Early manifestation of the retinoid-deficient defect may result also in formation of a cardia bifida while late manifestation results in development of a single dilated ventricle. In contrast, the extraembryonal vascular system of blood islands is well developed. Heart function as shown by the rate of heart beat is reduced in deficient embryos. We are now pursuing these studies at a more mechanistic level. In retinoid-deficient and control embryos the role of matrix proteins such as fibronectin and laminin is examined by using immunohistochemical staining of antibodies against such proteins. By doing so, we expect to obtain a better understanding of the molecular mechanisms of differentiation that are affected by retinoids.

Another mediator of cellular growth is the transforming growth factor TGF- β , originally identified in neoplastic cells; however, it is also widely distributed in a variety of nonneoplastic tissues such as blood platelets, placenta, kidney and activated peripheral blood lymphocytes. The presence of TGF- β in cells of hematopoietic origin indicates that it may play an intrinsic role in inflammation and tissue repair. Its capability to stimulate the formation of connective tissue during wound healing provides further evidence for this hypothesis.

We have studied, for 5 consecutive days, the induction of angiogenesis and activation of fibroblasts to produce collagen after subcutaneous injection of 200-800 ng/day TGF- β into the nape of the neck of newborn mice. Epidermal growth factor (EGF) in comparable dilutions and saline served as controls. Nape tissue was processed for light and transmission electron microscopy, using either neutral formalin or glutaraldehyde fixation and routine embedding and staining procedures (HE, Masson's trichrome stain, uranyl acetate, and lead citrate).

Within 48 hours, a dose- and time-dependent response resulted in the formation of a firm nodule at the injection site in 64 mice treated with TGF- β . No reaction was observed after EGF or saline treatment. In parallel, there exists a TGF- β -dependent proliferation of both small blood vessels and fibroblasts coinciding with activation of RER in fibroblasts and production of extracellular collagen. In conjunction with in vitro experiments demonstrating effects of TGF- β on collagen synthesis, our results indicate that TGF- β is a significant mediator of tissue repair.

Publications:

Heine, U. I., Roberts, A. B., Munoz, E. F., Roche, N. S., and Sporn, M. B.: Effects of retinoid deficiency on the development of the heart and vascular system of the quail embryo. Virchows Arch. [Pathol. Anat.] 50: 135-152, 1985.

Roberts, A. B., Sporn, M. B., Assoian, R. K., Smith, J. J., Roche, N. S., Wakefield, L. M., Heine, U. I., Liotta, L. A., Falanga, V., Kehrl, J. H. and Fauci, A. S.: Transforming growth factors-beta: Rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc. Natl. Acad. Sci. USA 83: 4167-4171, 1986.

Sporn, M. B., Roberts, A. B., Heine, U. I., Roche, N. S., Munoz, E. F., Smith, J. M., Smith, K. L., Dalton, S., Shealy, Y. F. and Dawson, M. I.: Retinoids and differentiation of cells of mesenchymal origin. In Saurat, J. H. (Ed.): Retinoids: New Trends in Research and Therapy. Basel, Geneva, S. Karger, 1985, pp. 35-39.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05487-01 LCC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Carcinogenesis and Mutagenesis by Fecapentaenes		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	J. M. Ward Chief, Tumor Pathology and Pathogenesis Section	LCC NCI
Others:	L. K. Keefer Chief, Chemistry Section	LCC NCI
	T. Anjo Visiting Associate	LCC NCI
	P. J. Donovan Chemist	LCC NCI
	J. M. Rice Chief	LCC NCI
COOPERATING UNITS (if any) Microbiological Associates, Inc., Bethesda, MD (M. Wenk); Stanford Research Institute, Palo Alto, CA (W. Bradford); Program Resources, Inc., Frederick, MD (A. W. Andrews)		
LAB/BRANCH Laboratory of Comparative Carcinogenesis		
SECTION Tumor Pathology and Pathogenesis Section		
INSTITUTE AND LOCATION NCI, NIH, Frederick, Maryland 21701-1013		
TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 0.2	OTHER: 0.8
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Fecapentaenes from human feces have been found to be direct-acting mutagens and are therefore prime candidates as human carcinogens, especially for the large bowel. A variety of in vitro studies by other investigators have demonstrated potent mutagenic effects of fecapentaene-12 (FP-12) in bacterial cells, although cell transforming activity in vitro is low and mutagenic activity for mammalian cells is weak. Thus, animal experiments have become necessary to characterize the in vivo toxic and carcinogenic effects. We first studied the purity and stability of FP-12 to determine the most effective handling procedures during animal exposure. The chemical was stable under argon in small aliquots but quickly decomposed after exposure to air. Vitamin E has shown promise for stabilizing fecapentaene solutions for use in carcinogenesis studies. Several types of animal experiments were performed. Skin painting studies in SENCAR mice revealed neither initiating activity nor complete carcinogenesis to the skin by repeated exposure. Intra-rectal and subcutaneous administration to mice and rats have not provided convincing evidence of the carcinogenesis of FP-12, although most studies are still in progress. In a preliminary but small intra-rectal mouse study, 1/15 mice had a small colonic carcinoma and 3 had foci of atypical colonic hyperplasia. Trans-placental mutagenesis by FP-12 in hamsters was not convincingly demonstrable, while mutagenesis in vivo in rats by the granuloma pouch assay was unequivocal but weak.		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI
L. K. Keefer	Chief, Chemistry Section	LCC	NCI
T. Anjo	Visiting Associate	LCC	NCI
P. J. Donovan	Chemist	LCC	NCI
J. M. Rice	Chief	LCC	NCI

Objectives:

To study the mechanisms of carcinogenesis of fecapentaene-12 in rats and mice. To study the potential carcinogenic activity of fecapentaene-12 in rodents.

Methods Employed:

To document the integrity of fecapentaene-12 during the course of biological experiments, quality control data were collected by high pressure liquid chromatography (HPLC) and ultraviolet spectrophotometry just before and immediately after each animal administration session was conducted for one rat experiment. FP-12 was studied in mice and rats by topical, intra-rectal and subcutaneous administration to evaluate its possible toxic and carcinogenic effects. In order to characterize initiating or complete carcinogenic effects of FP-12, the chemical was administered topically to SENCAR mice once (for initiation) or repeatedly (up to ten weekly exposures) for complete carcinogenesis. The skin tumor promoter, TPA, was subsequently administered to initiated mice. A major problem was the size of the delivered dose to the rodents because of the low solubility of FP-12 in conventional solvents. Initially all studies were performed with FP-12 in ethanol at a concentration of 1-2 mg/ml solvent and subsequently in DMSO at concentrations of 12-16 mg/ml. To study mutagenesis by FP-12 in vivo, pregnant Syrian hamsters, 12 days in term, were exposed to a single I.P. injection of FP-12 0.1 mmole/kg (25 mg/kg) dissolved either in DMSO (3 animals) or oil (2 animals). Twenty-four hours later all the fetuses of one mother were removed, pooled and dissociated, and frozen. This was considered one point. After a 5-day expression time, mutation induction was measured by resistance to diphtheria toxin. Alternatively, a granuloma pouch was generated in F344 rats by S.C. injection of air and FP-12 solution was injected into the pouch 2 days later, after which the pouch was excised, dissociated, and derived cells evaluated for mutation to 6-thioguanine (6-TG) resistance.

Major Findings:

Negligible decomposition was observed when special handling procedures were employed including use of a controlled atmosphere to ensure minimal contact of the solutions with oxygen, especially important during manipulation of concentrated stock solutions. These procedures were developed after examining data from stability studies showing that decomposition was extremely rapid (half-life 20 min.) when 6 mM fecapentaene-12 in 9:1 ethanol:dimethylsulfoxide was exposed to air. The fact that the half-life increased dramatically when this solution was diluted fivefold suggested that the decomposition was primarily a radical process initiated by autoxidation. This suggested, in turn, that fecapentaene solutions might be stabilized

by addition of appropriate radical scavengers. Promising results in this direction have been obtained with α -tocopherol. It should be noted that the fecapentaene-12 synthesized for these studies is actually a mixture giving at least four distinct peaks by HPLC. It has been suggested that the several components are stereoisomers of one another, presumably differing little in biological activity. Efforts are currently underway to separate these by preparative HPLC so that their spectral, chromatographic, and especially biological (e.g., mutagenic) properties can be individually examined. Hopefully, the structures of all components can be assigned from these data in the near future.

Whether SENCAR mice received an initiating dose of 0.56 mg in ethanol or 3 mg in DMSO, initiating activity could not be demonstrated after TPA exposure. A total dose of 5.6 mg in ten weekly exposures also did not induce tumors within 30 weeks. In contrast, 50 μ g of DMBA is highly effective as an initiator. Intra-rectal administrations to mice and rats were carried out with FP-12 in ethanol or DMSO. Seven to ten weekly exposures of total doses of 0.49-1.8 mg in ethanol and 16 mg in DMSO have not been associated with early mortality or clinical tumors in any mice or rats for up to 59 weeks. Rats receiving 16 mg are only in the tenth week, however. Seven weekly subcutaneous doses of FP-12 (total dose .49 mg) in mice did not produce tumors by 59 weeks.

The mutagenic activity in the Ames assay indicates that this compound is exceedingly mutagenic for *Salmonella*. When tested with TA 100 it yielded a value of 5300 revertants/microgram or 1325 revertants/nanomole; when compared with some of the compounds in Ames' original survey of published mutation data it can be seen to be among the most mutagenic of compounds; for instance the supermutagen MNNG yielded 1375 revertants/nanomole. In contrast FP-12, transplacentally administered to Syrian hamster fetuses, was found to be marginally mutagenic (1-3 X control). The granuloma pouch assay is a direct mutagenicity test which measures the extent of mutation induced in the cells of artificially elicited granulomatous tissue. FP-12 directly administered in the granuloma pouch in male F344 rats at a dose of 0.25 mg or 1 micromole/pouch was weakly mutagenic causing only a 31-fold increase over the spontaneous mutation rate for 6-TG resistance.

In summary, there is little evidence at this time for postulated carcinogenic activity of FP-12 in rodents. These negative findings may have occurred because of the low doses utilized, weak, carcinogenic activity of FP-12 (in relation to other direct-acting mutagens, such as nitrosoureas, and to DMBA), or insufficient periods of observation for tumor appearance. Studies are still in progress, specifically toward reconciling the discrepancy between bacterial mutagenesis by FP-12 and its lower apparent potency in mammalian systems.

Publications:

None

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05488-01 LCC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Mechanisms of Inorganic Carcinogenesis: Cadmium		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	M. P. Waalkes Senior Staff Fellow	LCC NCI
Others:	A. O. Perantoni Microbiologist	LCC NCI
	K. S. Kasprzak Visiting Scientist	LCC NCI
	S. Rehm Visiting Scientist	LCC NCI
COOPERATING UNITS (if any) Program Resources, Inc., Frederick, MD (C. Riggs, H. Issaq); Microbiological Associates, Inc., Bethesda, MD (M. Wenk)		
LAB/BRANCH Laboratory of Comparative Carcinogenesis		
SECTION Inorganic Carcinogenesis Working Group, Office of the Chief		
INSTITUTE AND LOCATION NCI, NIH, Frederick, Maryland 21701-1013		
TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) The mechanisms of cadmium carcinogenesis are under active investigation at the organismal, cellular and molecular levels. On the organismal level, cadmium injected subcutaneously in rats was found to induce tumors at the site of injection in an apparent dose-related fashion while cadmium-induced testicular tumors appeared to involve a threshold phenomenon. A possible association between cadmium treatment and prostatic tumors was also seen. Acutely, cadmium induced extensive necrotic lesions in the ovaries of hamsters, similar to what is seen in the testes. A series of experiments on the cellular level indicated that tolerance to cadmium was highly dependent on genetic capacity for the production of the metal-binding protein, metallothionein. Pharmacologic manipulations that resulted in hypomethylation of DNA also resulted in increased capacity for metallothionein synthesis and induction of tolerance to cadmium cytotoxicity in cell culture. On the molecular level, investigations into the nature of the metal-binding proteins of the rat testes showed that in this target tissue of cadmium carcinogenesis there is a clear absence of metallothionein, indicating that the capacity for the production of this protein may be an important determinant in the target site specificity of cadmium.		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

M. P. Waalkes	Senior Staff Fellow	LCC	NCI
A. O. Perantoni	Microbiologist	LCC	NCI
K. S. Kasprzak	Visiting Scientist	LCC	NCI
S. Rehm	Visiting Scientist	LCC	NCI

Objectives:

To study the mechanisms of carcinogenesis by cadmium by investigating the areas of target site specificity, tissue susceptibility determinants, and genetic basis for tolerance to cadmium toxicity.

Methods Employed:

Acute and long-term studies in animals involved the injection of cadmium salts into rats or hamsters followed by examination at necropsy for neoplastic and/or other pathologic changes. Metabolism of cadmium, including interactions with metal-binding proteins, induction of metallothionein, cellular uptake, etc., are determined by radioisotopic (gamma spectrometry) and standard analytical methods (atomic absorption spectrophotometry, HPLC, amino acid analysis, molecular sieve chromatography, ultracentrifugation).

Major Findings:

Dose response analysis in bioassay has provided evidence that the two major sites for induction of tumors by cadmium in rats, the injection site and the testes, show significant differences in response. The injection site showed dose-related trends in tumor formation, indicating a strict dependency on local concentration. Testicular tumors showed a more complex relationship to cadmium dosage, as a threshold dosage appeared necessary for tumor formation, indicating tissue-specific determinants. The acute phase necrosis of the testes induced by cadmium did not, however, appear to be the sole tissue susceptibility determinant as doses known to be necrotizing were not necessarily high enough for tumor formation. A possible link to cadmium induction of prostatic tumors in rats, a frequently suspected tumor site of humans exposed industrially to cadmium, was also detected. The tendency for increased formation of prostatic tumors occurred only with doses of cadmium low enough to avoid inducing acute necrosis of the testes with subsequent loss of function and atrophy of male sex accessory tissues (including prostate). Cadmium was found to have a remarkable acute effect on the ovaries of Syrian hamsters, inducing marked necrosis similar to that seen in testes. Similar susceptibility factors may be found in tissues of homologous embryonic origin.

On the cellular level tolerance to cadmium was found to be highly dependent on the genetic capacity for production of the metal-binding protein, metallothionein. A series of experiments indicated that agents that are known to hypomethylate DNA, such as 5-azacytidine, 5-aza-2'-deoxycytidine, dimethyl sulfoxide and butyric acid cause a marked increase in the cellular capacity for production of metallothionein upon cadmium exposure. Metallothionein is also synthesized more rapidly in response to cadmium in cells treated with these agents. The effect of all these agents

requires the active synthesis of DNA, indicating that pretranslational events could be very important. Enhanced capacity for cellular production of metallothionein results in the development of tolerance to the toxic effects of cadmium, indicating that the expressibility of the metallothionein gene is an important susceptibility determinant.

On the molecular level, studies into the nature of the metal-binding proteins of the rat testes revealed that there was a clear deficiency of metallothionein, the protein most frequently associated with tolerance to the adverse effects of cadmium. Although a protein of similar molecular weight that could bind cadmium was isolated from testes, this protein was not metallothionein and could be predicted to have a much lower affinity for cadmium binding on the basis of amino acid analysis. A deficiency of metallothionein in the rat testes may be a key determinant in the susceptibility of this tissue to the toxic and carcinogenic effects of cadmium.

Publications:

Boorman, G., Eustis, S., Waalkes, M. P. and Rehm, S.: Seminomas in rats. In Jones, T. C. (Ed.): Pathology of Laboratory Animals. New York, Springer-Verlag (In Press)

Kasprzak, K. S., Waalkes, M. P. and Poirier, L. A.: Effects of essential divalent metals on the carcinogenicity of nickel and cadmium. Biol. Trace Element Res. (In Press)

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Waalkes, M. P.: Effect of dietary zinc deficiency on the accumulation of cadmium and metallothionein in selected tissues of the rat. J. Toxicol. Environ. Health 18: 301-313, 1986.

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Waalkes, M. P. and Perantoni, A.: Isolation of a novel metal-binding protein from rat testes: Characterization and distinction from metallothionein. J. Biol. Chem. (In Press)

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Waalkes, M. P. and Wilson, M. J.: Enhancement of cadmium-induced metallothionein synthesis in cultured liver cells by butyric acid pretreatment. Toxicol. Lett. (In Press)

Waalkes, M. P., Wilson, M. J. and Poirier, L. A.: Reduced cadmium-induced cytotoxicity in cultured liver cells by 5-azacytidine pretreatment. Toxicol. Appl. Pharmacol. 81: 250-257, 1985.

Patents:

None

ANNUAL REPORT OF

THE LABORATORY OF EXPERIMENTAL CARCINOGENESIS CHEMICAL AND PHYSICAL CARCINOGENESIS PROGRAM DIVISION OF CANCER ETIOLOGY NATIONAL CANCER INSTITUTE

October 1, 1985 through September 30, 1986

The major mission of the Laboratory of Experimental Carcinogenesis (LEC) is to conduct innovative and productive research aimed at elucidating mechanism(s) of malignant transformation in human and animal cells by chemical carcinogens and other cancer causing agents. From its inception, this laboratory has sought to accomplish this goal by an integrated multidisciplinary approach to cancer research. The present structure of LEC combines expertise in the diverse disciplines of cell biology, chemical and viral carcinogenesis, molecular biology, protein and nucleotide chemistry, and computer science. A fundamental aspect of our multidisciplinary approach to cancer research stems from the strong belief that the neoplastic process must be studied at all levels of biological complexity ranging from the intact organism to defined in vitro systems in order to identify and characterize the critical cellular and genetic factors in cancer development. The selection of the rat liver model as the major experimental system to study the mechanism of chemical carcinogenesis is a reflection of this research approach.

During the last year, we began establishing the transgenic mouse system to study developmental regulation of genes that are intimately associated with the multi-stage process of carcinogenesis. The transgenic animal system provides a powerful method for perturbing the organization and expression of the mammalian genome. In germline transmission, every cell in the transgenic animal will carry the introduced foreign gene and the transgenic animal can be bred to generate numbers of offsprings. This opens up a new way to investigate tissue-specific and developmental-specific regulation of gene expression. Furthermore, the host animal can be monitored throughout normal development or oncogenesis. Our ultimate goal is to produce transgenic mouse (and later rat) lines that develop a tissue-specific tumor at a defined stage of development or at a specific age. In this context, the pattern of spatial and temporal expression of known oncogenes as well as other cancer-associated genes and their correlation to tumor formation can be studied in a developing animal. This system will allow us to conduct systematic investigations on the molecular and cellular changes during the development of a particular cancer as well as the role of individual oncogenes in that process. Furthermore, obtaining the transgenic mouse lines that develop a tissue-specific tumor at a defined stage of development or at a specific age would provide an invaluable resource for conducting experimental cancer treatments and/or chemoprevention studies.

The integrated research efforts of the LEC scientists have resulted in a number of unique accomplishments during the last year. These accomplishments in the major areas of investigation within LEC are summarized in the following sections.

Computer Analysis of Two-Dimensional Gels. The computer system, developed in this laboratory, for analyzing two-dimensional gels have been dubbed ELSIE III. It has been distributed to several laboratories in the United States and Europe. Some of the more significant advances in the past year are outlined below.

1) Findspots. A new spot-finding algorithm has been developed that combines both the thresholding method described by Vo et al. (Anal. Biochem. 112: 258, 1981) and the negative core detection method of Lemkin (Comp. Biomed. Res. 13: 571, 1981). First, the second derivative of the surface of the gel is calculated in both the X and Y directions using an elliptical least squares convolution template to both smooth the data and calculate the second derivative in one operation. Regions where the second derivative becomes negative, in both directions, identify one or more spots. These "negative core" regions are next subjected to a thresholding operation that searches for additional peaks within them. This algorithm improves the accuracy of the spot-finding programs without significantly increasing the number of false spots detected (about 1%).

2) Data organization. The data for each gel are kept in several files: e.g., one file contains the raw scan data, another the spot shapes, yet another the position and intensity information for each spot. In order to associate each data file with the proper two-dimensional gel and to keep the number of files stored in any directory at a minimum, a single, uniquely named subdirectory is created for gel and the data files for the gel are stored in that subdirectory. All programs that reference a gel are given only the name of that gel (the subdirectory). The user need not be concerned with the structure and names of the various subfiles that describe the gel; it is the program's responsibility to access the proper files and do the appropriate bookkeeping. This organization also allows us to change the names and structures of data files in a manner invisible to the users of the facility. Finally, the structure allows one to match two gels located anywhere on the computer system simply by specifying the complete path names of the two gel directories. A special file is created that incorporates the path-names of the gels and the sets of spots matched between the gels.

3) Analysis tools. Several program "tools" have been developed to aid in the analysis of processed data. A brief outline of some of the more significant programs follows: (a) Desaturate combines information about heavy and light exposures of a gel. A new set of files is created for the gel in which "saturated" data in the heavy exposure is replaced by the "unsaturated" information in the light. This effectively extends the dynamic range of the gels. (b) Manypairs matches together unmatched gels among a set of gels where some of them have been matched. For example, if we have four gels, A, B, C, and D, manypairs can be used to automatically match gel pairs B-C, B-D and C-D. (c) Plotcpm plots the intensity of groups of spots as a function of time for gels in an experiment. It can thus be used to plot the kinetics of synthesis of many individual proteins during the course of an experiment. (d) Findquant finds spots that are statistically different (T test) in intensity between two or more sets of gels. (e) Findqual uses statistical tests to find spots that are different qualitatively between two or more sets of gels; that is, spots that are intense in at least one set of gels and absent in at least one other.

4) Measures: picking what to measure to identify unknowns. One way to identify an unknown item is to measure its traits and compare the measurements with those of items the unknown might be. However, it may not be necessary to measure all of the unknown's traits in order to identify the unknown. For example, if a protein were known to be listed in the Protein Sequence Database (PSD -- Georgetown University Medical Center), it is often possible to identify the protein by measuring only some of its 20 amino acids.

2D-PAGE Analysis of Cellular Differentiation and Transformation. The computer-based two-dimensional gel electrophoresis analysis of total cellular protein patterns has been extensively used in studies on both cellular differentiation and transformation: (a) Studies on Ca^{2+} and TPA-induced differentiation of mouse epidermal cells have identified 11 proteins that were changed in the same direction for both differentiating agents. These results suggest that a common program of protein synthesis is induced by both Ca^{2+} and TPA and that these proteins are involved in epidermal differentiation; (b) Chemical transformation of Syrian hamster fetal cells (HFC) was analyzed by 2D-PAGE. Common qualitative changes were observed in seven neoplastic HFC lines. We have been able to identify polypeptide changes that are closely associated with both early morphological changes in the transformation process and the acquisition of tumorigenicity; (c) Studies employing the chemically induced rat hepatocarcinogenesis model have allowed us to identify sets of proteins common to both the preneoplastic and neoplastic stages that appear to be under coordinated regulation during the evolution of the neoplastic process; and (d) Analysis of polypeptide changes in biopsies from human mammary carcinomas revealed both qualitative and quantitative polypeptide differences when compared to normal breast tissue from the same individual.

Six cytosolic polypeptides were expressed in all malignant tissues (8 individuals) but not in normal tissue, while one polypeptide (55: pI 7.25/55 kDa) found in normal breast tissue was lost in tumor biopsies. More numerous quantitative changes were also observed. However, in contrast to the patterns that were observed during hepatocarcinogenesis, these changes were localized to a particular subset of polypeptides which were localized mainly in the pI range 6.0-7.0 and molecular weight ranges of 22-40 kDa. Comparison of polypeptides in this region revealed a general up-regulation of polypeptide expression in malignant tissues as compared to those from normal tissues. Included in this group of polypeptides is p24 (pI 6.15/24 kDa) which was expressed in greatest concentrations in tissues exhibiting the highest estrogen receptor (ER) content.

Oncogene Expression During Growth and Differentiation. We have investigated the expression of two oncogenes, c-myc and N-ras, associated with the human promyelocytic leukemia cell line HL60, both during terminal differentiation and growth inhibition by DFMO that is independent of terminal differentiation. We have associated the 8- to 10-fold decrease in c-myc transcripts with differentiation of the promyelocytes into mature monocytes. C-myc regulation also appears to be an early event of HL60 cells as evidenced by a burst of c-myc synthesis within the first hour of TPA treatment, followed by the rapid decline in the level of c-myc specific transcripts. This response was similar to that observed in mitogen stimulated B-cells, T-cells and normal fibroblasts although the HL60-induced level was neither as dramatic nor as long as that reported for normal cells. Although this response was reportedly associated with the cell cycle regulation of proliferation in normal cells, the level of c-myc-specific transcripts was found invariant during the HL60 cell cycle. In addition, unlike the mitogenic response of other agents, or normal resting cells, TPA-induced differentiation of HL60 cells resulted in no cellular proliferation or significant change in DNA synthesis, when compared to control or DFMO-treated cells during the first 24 hours, again supporting the association of c-myc with the differentiation state of the cell.

Chemical Transformation of Human Lymphoblastoid Cell Lines. In our attempt to define the nature of chemically-induced transition from a benign hyperproliferative to a malignant stage, we have succeeded in chemically transforming EBV-immortalized human lymphocytes into high grade immunoblastic lymphomas. We have also succeeded in transfecting the DNA of these lymphomas into NIH 3T3 cells and obtained transformed foci. Southern blot analysis of the genomic DNAs derived from these foci revealed presence of human DNA, yet none of the "classical" oncogenes were found in any of the transfected cells.

Chemical Hepatocarcinogenesis. One of the earliest and most reliable histochemical markers in chemically-induced hepatocarcinogenesis is gamma-glutamyl-transpeptidase (GGT). We have obtained a genomic clone for this important marker, making it possible to study, in detail, the regulation of this gene during the carcinogenic process. A major cell surface receptor characterizing both human and rat hepatocytes is the asialoglycoprotein receptor (ASGP). This receptor is a transmembrane glycoprotein which mediates the binding and internalization of glycoproteins with terminal galactose or N-acetyl-galactosamine residues, and the receptor function has been associated with nonproliferating, differentiated hepatocytes. Using immunohistochemical methods we have shown the lack of cell surface ASGP receptors in focal preneoplastic areas in rat liver during the initial stages of chemically induced hepatocarcinogenesis. These receptor deficient areas are superimposed with both glucose-6-phosphatase-deficient and GGT-positive areas in serial liver sections, indicating that this receptor is an early marker protein for cell surface alterations during hepatocarcinogenesis. We have also shown that ASGP cell surface receptors are decreased and/or absent in actively proliferating hepatocytes in vivo, such as in developing, regenerating or neoplastic liver. In addition, we have demonstrated that both acute and chronic treatment with phenobarbital, a powerful promoter of chemical hepatocarcinogenesis, decreases the cell surface receptors. These data are in agreement with certain ASGP ligand binding studies and suggest that, in addition to its role in the endocytosis of deasialyated glycoproteins, the ASGP receptor may also participate in receptor-mediated cell-cell communication and recognition.

Northern blot analysis, however, indicates that the decrease in cell surface ASGP receptor in the actively proliferating in vivo hepatocyte is the result of a post-transcriptional effect(s), since specific ASGP receptor transcripts are not decreased in fetal, neonatal, regenerating or phenobarbital-treated liver, as well as in preneoplastic nodules and primary hepatocellular carcinomas.

Metabolic Processing of Chemical Carcinogens. Metabolic processing of chemical carcinogens by human tissues have been studied. Two distinct phenotypes, slow and fast metabolizers, were observed for both metabolic activation and detoxification of the model carcinogen, 2-acetylaminofluorene (AAF), in human liver microsomes from 28 individuals. We observed that individuals who were fast activators of the carcinogen were, in most cases, also fast detoxifiers of the chemical. However, different phenotype patterns exist, suggesting that fast activators of a toxin need not also be phenotyped as fast detoxifiers. The metabolic activation of the amino acid pyrolysate Trp-P-2 to a mutagen by the same human liver samples was also polymorphic and there was a highly significant correlation between the N-hydroxylation of AAF and the mutagenicity of Trp-P-2. Studies in this area may help to understand whether certain individuals are predisposed to a higher rate of chemically-induced cancer.

Differential Effects of Transforming Growth Factor-Beta on Proliferation of Normal and Malignant Rat Liver Epithelial Cells in Culture. Transforming growth factors (TGF-beta) have been shown to cause stimulatory and inhibitory effects on cellular growth in a variety of normal and neoplastic cells. The nature of the inhibitory effects of TGF-beta on proliferation of different cell types is at present unclear. We have used freshly isolated rat hepatocytes, a normal diploid rat liver epithelial cell line (FNRLM) and a subline (AFB) derived from it by transformation in vitro by aflatoxin beta-1 (AFB₁) to study the nature of TGF-beta-induced growth inhibition and its alteration following chemically-induced neoplastic transformation. TGF-beta had a vastly different effect on proliferation of normal rat liver epithelial cells (both freshly isolated hepatocyte and FNRLM cells) compared to AFB₁-transformed cells. TGF-beta at 2 pg/ml caused 83% inhibition of colony formation of FNRLM, whereas the growth of AFB cells was unaffected by TGF-beta at concentrations as high as 10 mg/ml. A parallel dose-dependent inhibition of DNA synthesis was observed in AFB cells. Furthermore, TGF-beta had no effect on anchorage independent growth of either FNRLM or AFB cells. TGF-beta-induced inhibition of the FNRLM cells was irreversible in nature since treated cells were unable to proliferate and form colonies upon removal of TGF-beta from the medium. Also, FNRLM cells showed, after 4 days in the presence of 20 pg/ml of TGF-beta, morphological changes characterized by cytoplasmic hypertrophy and the formation of abundant liposomal derivatives, some of which resembled lipofuscin. The finding that TGF-beta caused a high degree of irreversible inhibition of FNRLM cells emphasized the need for caution in interpreting data from inhibition studies, since most assays presently employed are designed for assessing growth stimulation in vitro and do not adequately distinguish between the possible cytotoxic and/or cytostatic action of growth inhibitors.

Chemical Structure Studies on Gamma-Glutamyl Transpeptidase (GGT). Gamma-glutamyl transpeptidase (GGT) is a membrane-bound glycoprotein enzyme of unknown structure that is involved in the degradative metabolism of glutathione and the uptake of some amino acids. In rodents, GGT activity is especially high in fetal liver and in adult kidney but not in adult liver. The low liver enzyme activity is inducible by certain drugs and carcinogens such as azo dyes. For example, elevated levels of GGT are found in most hepatomas. This enzyme is thus expected to be a marker of neoplastic transformation, especially in hepatoma cells. It is also known that there are structural differences between the enzymes found in tumorigenic tissue and the corresponding normal tissues. We have purified to homogeneity the active heterodimeric enzyme from adult rat livers. A fast preparative HPLC method was developed to separate the two enzymic subunits under acidic conditions. 2D-PAGE was found to resolve the active enzyme into at least 18 components. Seven components with apparent molecular weight of 23-26,000 comprised the light subunit, and 11 components with apparent molecular weight of 51-53,000 comprised the heavy subunit. Immunoblot analysis of 2D-PAGE showed that all of these components are immunoreactive with a mixture of the two antibodies generated separately against the light and heavy subunits, demonstrating that they are all valid constituents of the enzyme complex. N-terminal amino acid sequencing of the separated subunits of the enzyme yielded, for the first time, sequence information for the first 32 residues of the heavy chain and for 36 residues of the light subunit. The sequence information allowed us the construction of oligonucleotide probes for cloning of the GGT gene. Whereas the 2D-PAGE

results indicate considerable heterogeneity for the active enzymes, the sequencing information, at least to the extent determined here, confirms earlier observations that the heterogeneity does not reside in the amino acid sequence of the enzyme, but rather in the attached carbohydrate chains.

Hepatic Proliferation Inhibitor (HPI) Structural Efforts. Previous work shows that liver contains an endogeneous growth inhibitory substance that acts on normal liver epithelial cells in vitro in a reversible manner, but is ineffective on hepatoma (transformed) cell lines. In order to explore the significance of this growth regulatory substance, we first developed a semiautomated assay system for measurement of cell proliferation based on DNA content in the cells. In this assay, cell chromatin-enhanced dye fluorescence was shown to be a linear function of the number of cells or cell nuclei per sample wells. This fluorometric assay was useful for the direct assay of the influence of growth factors on cell proliferation. This convenient assay also correlates well with the thymidine incorporation method of assay for DNA synthesis in cell cultures. The bioassay was utilized in the development of a preparative isolation scheme for the rat hepatic proliferation inhibitor. Starting with a previous preparative scheme for HPI, a new procedure has been developed for the purification employing modern chromatographic methods. One-dimensional gel electrophoresis on the purified material to date show three components. The components are being characterized for biological activity and amino acid sequence following gel elution techniques. Some functional and structural studies have been carried out on the partially purified HPI. It is acid stable and reasonably heat stable to 50°C. It is unstable to organic solvents; it is not a glycoprotein, appears to be a single chain polypeptide of approximately 20,000 daltons in size, and it is different from transforming growth factor-beta, which is also active as an inhibitor of normal liver epithelial cell proliferation.

Development of Methodologies for the Isolation and Chemical Characterization of Proteins from Two-Dimensional Polyacrylamide Gels. The computer-assisted two-dimensional gel electrophoresis methodology developed within this Laboratory has provided a highly sensitive procedure capable of resolving large numbers of proteins within a sample. The expression levels of a number of proteins were found to be different when compared in normal, preneoplastic and neoplastic rat livers. The identification and structural definition of these proteins is an important prerequisite before determining their possible functional role. Microanalytical, chemical and spectrometric methods are being developed for these purposes. Thus, methods are developed using radiolabelled standard proteins to electroelute and to acid extract sub-nanomolar amounts of proteins from polyacrylamide gels. Methods are developed to purify these gel-recovered proteins in order to make them suitable for N-terminal amino acid sequencing. Fast atom bombardment (FAB) mass spectrometry has been applied for structural studies of synthetic peptides related to the oncogenic ras p21 protein. Molecular weight information has been obtained to four significant figures for one of the 34 residue peptides. FAB mass spectrometry is being adapted to optimize the sequencing information obtainable on tryptic peptide digests of standard molecules. Derivation by group-specific reagents, such as p-bromobenzoylchloride, were found to simplify the peptide spectra such that sequence information can be extracted from the data easier. The FAB spectra of a class of N-terminally blocked peptides, the pyroglutamates, were examined, since the classical Edman method is ineffective in sequencing these. Results indicate that the spectra

allow recognition of the pyroglutamate functionality on the peptide and that there are several clear-cut trends, depending on the sequence information, according to which of these peptides fragment in the mass spectrometer.

Guanosine Triphosphate Binding Site of ras Proteins by Physiochemical Techniques.

Position 12 is one of the important mutation sites in the ras family of viral oncogenic proteins, when comparison is made to their analogous cellular non-transforming and transforming p21 proteins in terms of physiochemical properties of these molecules. We have chemically synthesized both the glycine-containing and the valine-containing N-terminal 34 residue peptide segments encompassing position 12 of the ras protein. To date, we have characterized the glycine-containing peptide using circular dichroism of P-31 nuclear magnetic resonance spectroscopy and equilibrium dialysis methods. Our results indicate that both GTP and ATP form complexes with the gly-peptide indiscriminately and that a longer segment, possibly the intact 189 residue p21 protein chain, is required for the assumption of the proper tertiary structure such that binding occurs exclusively with GTP and GDP. Furthermore, circular dichroism experiments indicate that addition of the nucleotide to the gly-peptide induces conformational changes in the peptide. When the amino acid at position 12 is valine, such conformational changes may be impeded. We are proceeding to test this hypothesis.

Viral-Induced Mammary Tumorigenesis. In earlier studies, sequencing the mouse mammary tumor virus led to the discovery of a fourth gene in MMTV, a finding that prestaged the more recent demonstration of similar genes in the HTLV viruses. All other replication-competent retroviruses encode only gag, polymerase, and envelope functions. Fragments of this putative protein have been expressed in bacteria, confirming the existence of the fourth gene. Current investigations focus on efforts to elucidate the function of this gene and its potential involvement in viral-induced mammary oncogenesis.

Characterization of H-v-ras. Cell lines have been developed that are conditionally transformed by the v-ras^H oncogene. In these cells, the v-ras^H gene is driven from the MMTV promoter; as a result, levels of the p21 gene product are subject to regulation by glucocorticoids. Two rare transfectants have been characterized in which the level of p21 protein is sufficiently low in the absence of hormone that the cells revert to normal phenotype in the absence of hormone. Studies are underway to identify potential changes in gene expression during this phenotype switch utilizing the high-resolution two-dimensional gel technology developed in the Laboratory. The availability of a system for conditional expression of the transformed phenotype has stimulated considerable interest, and many groups have applied this approach in the study of different oncogenes, as well as other cellular functions.

A Chromatin Structure at the MMTV Promoter. A major project has been initiated to characterize chromatin structure at the MMTV promoter, and to determine the potential role of nucleoprotein structures in hormone action. We have discovered that nucleosomes are phased in the regulatory region upstream of the MMTV cap site, indicating that the trans-acting soluble factors that act at this locus interact with a highly ordered chromatin structure. The implication for the mechanism of transcriptional regulation is one of the major current projects.

In addition to their intramural research efforts that have been summarized above, investigators within the LEC serve on editorial boards of major journals in their fields and are involved as consultants or advisors on various national and international committees in the area of chemical and biological carcinogenesis. Furthermore, the LEC scientists participate to a considerable degree in collaborative efforts with scientists both within the NCI and throughout the country, and the international scientific community.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP04986-09 LEC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Molecular Basis of Steroid Hormone Action		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Gordon L. Hager	Head, Hormone Action & Oncogenesis Section LEC NCI
Others:	Michael Cordingley	Visiting Associate LEC NCI
	Anna Riegel	Visiting Fellow LEC NCI
	Ronald G. Wolford	Microbiologist LEC NCI
	Diana S. Berard	Microbiologist LEC NCI
COOPERATING UNITS (if any) Laboratory of Chemistry, NIADDK, NIH (S. Simmons)		
LAB/BRANCH Laboratory of Experimental Carcinogenesis		
SECTION Hormone Action and Oncogenesis Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> The mouse mammary tumor virus (MMTV) has emerged as the leading model system for the study of gene regulation by steroids at the transcriptional level. We have established that the glucocorticoid responsive regulatory element (HRE) for the MMTV promoter is completely encoded within the long terminal repeat (LTR) of the virus. The v-ras-H gene of Harvey murine sarcoma virus (HaMuSV) was fused to the complete MMTV-LTR promoter. After introduction of this chimeric DNA into NIH 3T3 cells, steady state levels of p21 protein (the product of the v-ras-H gene) were responsive to regulation by glucocorticoids. Subsequently we found that the MMTV promoter is subject to strong activation by an enhancer element derived from the U3 region of the HaMuSV, both in transfection efficiency of LTR-v-ras-H fusion genes, and in transient expression of LTR-chloramphenicol acetyltransferase fusion genes. We discovered that although the activity of the LTR is highly stimulated in the presence of glucocorticoids, the enhancer does not efficiently activate transcription from the LTR when glucocorticoids are not present in the tissue culture medium. In fact, transformation efficiency by the linked v-ras-H gene is depressed compared to that obtained with the activating element driving transcription from a nonregulated promoter. Functional analysis of LTR deletion mutations assayed both in a focus-forming assay or transient assay with CAT fusion genes have localized the sequences required for the hormonal response. Furthermore, mutations which abolish hormone responsiveness also destroy the negative influence which the intact LTR exerts on transcriptional activation by the upstream enhancer. Finally, experiments utilizing episomally mobilized MMTV LTR fusion genes have established that a labile protein is responsible for mediating the negative modulation of enhancer activity that we have observed, and further that it mediates its activity by interaction with sequences closely associated with the hormone regulatory element of the LTR. </p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Gordon L. Hager	Head, Hormone Action & Oncogenesis Section	LEC NCI
Michael Cordingley	Visiting Associate	LEC NCI
Anna Riegel	Visiting Fellow	LEC NCI
Ronald G. Wolford	Microbiologist	LEC NCI
Diana S. Berard	Microbiologist	LEC NCI

Objectives:

- (1) Analysis of hormone regulated transcription of mouse mammary tumor virus (MMTV).
- (2) Determination of the mechanism of hormone action in the up-regulated MMTV system; extension of the investigation of glucocorticoid regulation into systems where the expression is down-regulated.
- (3) Application of tools developed for the study of hormone action to the study of other transcriptional regulatory systems important in cell growth.

Methods Employed:

Molecular chimeras between the MMTV long terminal repeat (LTR) and the v-ras gene of Harvey murine sarcoma virus (HaMuSV) are used in a hormone-dependent transfection assay to probe the regulatory regions involved in hormone induction of MMTV expression. Similar fusions between the LTR and the chloramphenicol acetyl transferase (CAT) gene from the bacterial Tn9 transposon will be tested in a transient expression assay. Deletion analysis of molecular chimeras will be performed to localize these regions.

Utilizing the S1 nuclease or mung bean nuclease mapping techniques, probes available from molecular clones of MMTV will be utilized to analyze steroid-dependent MMTV regulation.

Transcriptional promoters with negative response to glucocorticoids (proopiomelanocortin), and positive response to other hormones (androgens) will be engineered into the v-ras transformation system to test for transfer of regulation.

Major Findings:

This series of experiments have succeeded in identifying sequences within the genome of MMTV which confer hormone regulation of transcription initiation. We have defined a short region between 107 and 225 base pairs upstream from the cap site which is essential for the hormone response. Identification of the hormone regulatory sequences was of paramount importance for the study of fundamental aspects of eukaryotic gene regulation since glucocorticoid regulatory elements are one of only a handful of transcriptional elements for which a transacting regulatory factor, the glucocorticoid receptor in this case, have been characterized.

Furthermore experiments with the HaMuSV enhancer linked to the MMTV LTR have revealed a negative modulation of transcription associated with the unstimulated LTR. This negative modulation appears to be caused by a labile protein which mediates its effect through sequences in or closely associated with the hormone regulatory element. These observations may have wide ranging implications both for steroid regulated promoters and other regulated eukaryotic genes. It may be that both positive and negative modulatory factors are involved in regulation of transcription initiation at many eukaryotic gene promoters.

Publications:

Cordingley, M. G., Richard-Foy, H., Lichtler, A. and Hager, G. L.: A transacting factor negatively regulated transcription at the MMTV LTR. In Granner, D. K., Rosenfeld, G. and Chang, S. (Eds.): Transcriptional Control Mechanisms: UCLA Symp. on Molec. and Cell Biology, New Series, New York, Alan R. Liss, Inc., Volume 52 (In Press).

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05261-05 LEC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Metabolic Processing of Chemical Carcinogens		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: Snorri S. Thorgeirsson Chief LEC NCI		
COOPERATING UNITS (if any) National Institute of Public Health, Oslo, Norway (E. Dybing); Dept. of Clinical Pharmacology, Royal Postgraduate Medical School, London, England (D. S. Davies), Department of Pharmacology, The University of Western Australia (R. F. Minchin).		
LAB/BRANCH Laboratory of Experimental Carcinogenesis		
SECTION Chemical Carcinogenesis Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 0.1	PROFESSIONAL: 0.1	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The research is, at present, focused on the relative roles of metabolic activation and detoxification in determining both mutagenic and carcinogenic potential of aromatic amines and amides. Two distinct phenotypes, slow and fast metabolizers, were observed for both metabolic activation and detoxification of the model chemical carcinogen, 2-acetylaminofluorene (AAF), in the human liver microsomes from 28 individuals. We observed that individuals who were fast activators of the carcinogen were, in most cases, also fast detoxifiers of the chemical. However, different phenotype patterns exist suggesting that fast activators of a toxin need not also be phenotyped as fast detoxifiers. The activation of the amino acid pyrolysate Trp-P-2 to a mutagen by the same human liver samples was also polymorphic and there was a highly significant correlation between the N-hydroxylation of AAF and the mutagenicity of Trp-P-2. There was no correlation between the N-hydroxylation of AAF and 4-hydroxylation of debrisoquine in these human samples.</p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Snorri S. Thorgeirsson

Chief

LEC NCI

Objectives:

The main objectives of the project are (1) to define, in an intact cell system, the metabolic processing of chemical carcinogens, especially carcinogenic aromatic amines and amides, and to identify the metabolic pathways that are responsible for activation and detoxification of these compounds; (2) to study the mechanism whereby carcinogenic aromatic amines and amides cause mutations and other types of genotoxicity in both microbial and mammalian cell systems; and (3) to characterize the kinetic parameters for both metabolic activation and detoxification reactions for carcinogenic aromatic amines.

Methods Employed:

The principal methods are (1) bacterial and mammalian culture techniques, (2) differential centrifugation, (3) enzyme assays, (4) recording spectrophotometry, and (5) high pressure liquid chromatography.

Major Findings:

Two distinct phenotypes, slow and fast metabolizers, were observed for both metabolic activation and detoxification of the model chemical carcinogen, 2-acetylaminofluorene (AAF), in the human liver microsomes from 28 individuals. We observed that individuals who were fast activators of the carcinogen were, in most cases, also fast detoxifiers of the chemical. However, different phenotype patterns exist suggesting that fast activators of a toxin need not also be phenotyped as fast detoxifiers. The activation of the amino acid pyrolysate Trp-P-2 to a mutagen by the same human liver samples was also polymorphic and there was a highly significant correlation between the N-hydroxylation of AAF and the mutagenicity of Trp-P-2. There was no correlation between the N-hydroxylation of AAF and 4-hydroxylation of debrisoquine in these human samples. Kinetic parameters were determined for N- and C-hydroxylations of AAF in human, rat and rabbit liver microsomes. The N-hydroxylation of AAF was best described by a single enzyme system with high affinity and low capacity for all the species, and only one cytochrome P₄₅₀ isoenzyme (Form 4) out of six highly purified rabbit liver isoenzymes was capable of catalyzing the N-hydroxylation of AAF. 7-Hydroxylation of AAF, a major detoxification pathway for this compound, was consistent with a two enzyme system in all the species, displaying a high affinity and relatively low capacity and low affinity and high capacity components. The K_m values for 7-hydroxylation of AAF by these two enzyme systems differ by approximately three orders of magnitude, indicating that the two families of isoenzymes responsible will predominate at vastly different substrate levels. Genotoxicity of several heterocyclic amines that are found in pyrolysates of amino acid and proteins and have been isolated from broiled fish and beef was observed in the *Salmonella*/hepatocyte system. These genotoxic effects were modulated by agents affecting the

activity and composition of the cytochrome P₄₅₀ system. Both N-hydroxy-2-acetylaminofluorene and N-acetoxy-2-acetylaminofluorene induced DNA damage in rat and mouse livers both in vivo and in vitro as measured by alkaline-induced DNA strand breaks. A positive correlation was found between the extent of DNA strand breaks and the formation of either guanine C-8 AAF or guanine C-8 AF adducts.

Publications:

Hayashi, S., Moller, M. and Thorgeirsson, S. S.: Genotoxicity of heterocyclic amines in the Salmonella/hepatocyte system. Jpn. J. Cancer Res. 76: 835-845, 1985.

Minchin, R. F., McManus, M. E., Boobis, A. R., Davies, D. S. and Thorgeirsson, S. S.: Polymorphic metabolism of the carcinogen 2-acetylaminofluorene in human liver microsomes. Carcinogenesis 6: 1721-1724, 1985.

Minchin, R. F., McManus, M. E., Thorgeirsson, S. S., Schwartz, D. and Boyd, M. R.: Metabolism of 2-acetylaminofluorene in isolated rabbit pulmonary cells: Evidence for the heterogeneous distribution of monooxygenase activity in lung tissue. Drug Metab. Dispos. 13: 406-411, 1985.

Vu, V. T., Grantham, P. H., Roller, P. P., Hankins, W. D., Wirth, P. J. and Thorgeirsson, S. S.: Formation of DNA adducts from N-acetoxy-2-acetylaminofluorene and N-hydroxy-2-acetylaminofluorene in rat hemopoietic tissues in vivo. Cancer Res. 46: 233-238, 1986.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05262-05 LEC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Cellular Evolution of Chemically Induced Murine Hepatomas		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Ritva P. Evarts	Veterinary Medical Officer LEC NCI
Others:	Snorri S. Thorgeirsson Elizabeth R. Marsden	Chief LEC NCI Biologist LEC NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Experimental Carcinogenesis		
SECTION Chemical Carcinogenesis Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	1.2	1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The goal of this project is to determine the sequence of cellular events in hepatocarcinogenesis. <u>In situ</u> hybridization of albumin c-DNA and RNA probes for the albumin and <u>alpha-fetoprotein</u> genes to the hepatocytes in preneoplastic foci and in hepatocellular carcinomas revealed a decreased expression. Albumin expression is also decreased in the centrilobular areas of liver after phenobarbital administration. <u>alpha-Fetoprotein</u> is not expressed in early pre-neoplastic lesions but occasionally it is expressed in groups of cells inside the neoplastic nodule. We were able to transplant hepatocytes obtained from the individual nodules by needle biopsies to the anterior chamber of the rat eye. In order to study events in chemical hepatocarcinogenesis <u>in vitro</u>, a tissue culture system, which preserves the differentiated state of hepatocytes, is a necessity. We have demonstrated maintenance of asialoglycoprotein receptor, which we regard as an excellent marker for fully differentiated hepatocyte, for an extended period of time, when hepatocytes were cocultured with rat liver epithelial cell line. Treatment of rat liver epithelial cells with TGF-beta increased the number of binucleated cells, the expression of albumin and the size of these cells.</p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Ritva P. Evarts	Veterinary Medical Officer	LEC NCI
Snorri S. Thorgeirsson	Chief	LEC NCI
Elizabeth R. Marsden	Biologist	LEC NCI

Objectives:

The object of this project is to characterize the cellular evolution of chemically-induced murine hepatomas and to identify the contributions of different genes and gene products to the phenotypes of the transformed cells. Topics under investigation are: (1) identification of phenotypic changes during initiation, promotion and progression; (2) both temporal and cell-specific distribution of gene transcripts among normal, preneoplastic and neoplastic liver cells; (3) contribution of liver tumor promoters to gene expression and distribution of transcriptionally active genes.

Methods Employed:

(1) In situ hybridization for spatial localization of mRNA. (2) Immunocytochemistry for localization of cell surface receptors using immunoenzyme or immunofluorescence techniques. (3) Perfusion technique for separation of hepatocytes. (4) Methods for coculturing primary hepatocytes with liver epithelial cell line.

Major Findings:

(1) In situ hybridization technique combines histology with methods used in molecular biology. It makes a high resolution mapping of intracellular mRNA possible. We have optimized the conditions for successful in situ hybridization of ³H-labeled albumin and alpha-fetoprotein probes using either double-stranded or single-stranded probes. Albumin is less expressed in preneoplastic foci and in hepatocellular carcinomas. Albumin expression is also decreased in the centrilobular area of liver after phenobarbital administration. alpha-Fetoprotein is not expressed in early preneoplastic foci. However, occasionally alpha-fetoprotein is expressed in groups of cells inside the neoplastic nodule. Using single-stranded RNA probes prepared in the Gemini system, we are extending our research to include RNA probes for the following oncogenes: c-myc, c-raf and c-fos riboprobes. In addition the expression of asialoglycoprotein receptor, tyrosine aminotransferase and tryptophan oxygenase will be studied in early and late preneoplastic lesions in liver by using either Solt-Farber or Peraino's protocols for tumor production. We are using in situ hybridization technique in conjunction with histochemical and immunohistochemical methods, thus relating cellular structure to the molecular aspects of gene expression within individual cells. Our goals are: (a) to gain insight to the target cells in chemical hepatocarcinogenesis, (b) to elucidate gene expression of target cells during chemical carcinogenesis, and (c) to study putative cell lineages in experimental hepatocarcinogenesis.

(2) Transplantation of isolated normal, preneoplastic and neoplastic hepatocytes into the anterior chamber of the rat eye allowed these cells to survive for an extended period of time. With this methodology the hepatocytes are placed in a different microenvironment and possibly are released from the normal mitoinhibitory or stimulatory influence of unaltered hepatocytes. It is well known that many of the hyperplastic liver nodules will not progress to hepatocellular carcinomas and some of them will become remodelled to apparently normal phenotypes. By using needle biopsies of different liver nodules from the same liver and subsequently performing histochemical, immunohistochemical and in situ hybridization studies of the transplanted nodules, we hope to elucidate factors that are important for the progression of hyperplastic nodules to hepatocellular carcinomas.

(3) Hepatocytes in the primary culture lose their differentiated state in a few days as evidenced by decrease of albumin expression, loss of glucose-6-phosphatase, appearance of gamma-glutamyltranspeptidase and absence of functional asialoglycoprotein receptor. We have demonstrated the existence of asialoglycoprotein receptor up to 3 weeks in culture when hepatocytes are cocultured with rat liver epithelial cell line. In addition these cells maintain the expression of glucose-6-phosphatase for extended periods of time and are gamma-glutamyltranspeptidase negative.

To optimize the conditions for hepatocyte cocultures, experiments on defined media are under investigation.

(4) Morphological changes in the rat liver epithelial cell line have been observed when these cells in tissue culture have been treated with TGF-beta. A possible change of these undifferentiated cells towards fully differentiated hepatocyte is evidenced by increased size of the cells, expression of albumin and by the presence of binucleated cells after TGF-beta treatment.

Publications:

Evarts, R. P., Marsden, E. R. and Thorgeirsson, S. S.: Modulation of asialoglycoprotein receptor levels in rat liver by phenobarbital treatment. Carcinogenesis 6: 1767-1773, 1985.

Evarts, R. P., Raab, M., Marsden, E. and Thorgeirsson, S. S.: Histochemical changes in livers from portocaval-shunted rats. JNCI 76: 731-738, 1985.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER ZO1CP05263-05 LEC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Computer Analysis of Carcinogenesis by Two-Dimensional Gel Electrophoresis		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Mark J. Miller	Senior Staff Fellow LEC NCI
Others:	Arthur David Olson	Computer Programmer LEC NCI
	Snorri S. Thorgeirsson	Chief LEC NCI
	Peter J. Wirth	Expert LEC NCI
	Timothy Benjamin	Chemist LEC NCI
	Dolores M. Schwartz	Biologist LEC NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Experimental Carcinogenesis		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.) <p>The main objective of this project is to study the mechanism of carcinogenesis using quantitative two-dimensional gel electrophoresis. Research is, at present, focused on the following areas: (1) continued development and improvements on the computer system (dubbed ELSIE III) currently used to automatically analyze two-dimensional gels, and (2) use of ELSIE III to analyze experiments requiring computerized analysis of two-dimensional gel electrophoretograms. In the past year emphasis has been placed on developing software tools to aid the investigator in identifying spots of interest in an experiment. Statistical tests have been incorporated into various programs designed to search through sets of matched spots. These tests can be used to search for spots that may vary quantitatively or qualitatively over the course of an experiment. Once such spots are flagged, interactive computer graphics can be used to examine the spots and confirm their relevance to the experiment. The system has been used in several studies in this and other laboratories. One study involves the detailed quantitative evaluation of modulation in the rates of protein synthesis in the rat hepatoma cell line, H4-II-E (Reuber cells). Several single-cell-derived cultures of H4-II-E were prepared and grown under identical conditions. Cells were labeled as soon as possible after the cultures were established and two-dimensional gels were run on a total cell lysate. Quantitative analysis showed that 10% or more of the proteins demonstrate statistically significant modulation in their rates of synthesis when compared from culture to culture. These differences were generally on the order of 50%, although changes of as much as fourfold were detected. We conclude that the rates of synthesis of many polypeptides can vary significantly in a culture as a result of very subtle (and apparently uncontrollable) variations in the environment of cells and that the ELSIE III system is capable of detecting these changes.</p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Mark J. Miller	Senior Staff Fellow	LEC NCI
Snorri S. Thorgeirsson	Chief	LEC NCI
Peter P. Wirth	Expert	LEC NCI
Arthur D. Olson	Computer Programmer	LEC NCI
Timothy Benjamin	Chemist	LEC NCI
Dolores M. Schwartz	Biologist	LEC NCI

Objectives:

The main objective of this project is to study the mechanism of chemical carcinogenesis by employing the technique of quantitative two-dimensional gel electrophoresis of total cellular proteins. Since this technique allows for the simultaneous separation of total cellular polypeptides on a single polyacrylamide gel, it is possible to follow changes in the rate of synthesis of individual proteins as well as changes in the total protein patterns as the cell undergoes malignant transformation. Our aim is to identify and characterize those proteins that are associated with the transformed phenotype.

Methods Employed:

The principal methods employed are: (1) two-dimensional gel electrophoresis, (2) tissue culture techniques, (3) computer-based quantitation of autoradiograms, and (4) radioisotope measurements.

Major Findings:

From its inception, the major objective of the Laboratory's computer facility has been to further expand and develop the two-dimensional gel analysis system in order to facilitate the use of this important research technique in the analysis of the neoplastic process.

A. Advances in the Computerized Analysis of Two-Dimensional Gels. The computer system, developed in this Laboratory for analyzing two-dimensional gels, has been dubbed ELSIE III. It has been distributed to several other laboratories in the United States and Europe. In the past year emphasis has shifted toward the analysis of experiments utilizing quantitative two-dimensional gel electrophoresis. Several projects in this laboratory make use of the ELSIE III system in the analysis of experiments. Most of these are discussed elsewhere in this report (but see below). Considerable effort was placed in finding statistical tests that will give useful results in the search for spots that vary quantitatively or qualitatively over the course of an experiment. Generally, a modified version of the T test has proved most useful in finding such spots. This statistical test system has been incorporated into several of the programs, including LISTGROUPS, which lists the information about spots matched across multiple gels in a hard copy, tabular form, and VIEWGROUPS, which allows investigators to examine the spots directly using interactive computer graphics. In addition, there have been general improvements in the core analysis programs

that are responsible for finding spots, measuring each spot's density and matching together the spot patterns of different gels.

B. Analysis of Individual Polypeptides in the Hepatoma Cell Line H4-II-E. In order to examine the degree of heterogeneity in polypeptides synthesized in a transformed cell line, several single-cell subclones were isolated from the rat hepatoma cell line, H4-II-E. Three of these were analyzed in some detail by quantitative two-dimensional gel electrophoresis. Out of a total of 746 polypeptide spots examined across the three subclones, only two were found to be qualitatively different in the sense that they were not detectable in at least one of the subclones. On the other hand, on average, 9.5% of the spots showed statistically significant quantitative differences when one subclone was compared to any other subclone. Most of these quantitative changes were on the order of 50%, although some varied by fourfold or more.

A second round of subclones were isolated from the primary subclones and these were also examined using quantitative 2D-PAGE. No consistent qualitative differences were detected in the 2D-PAGE patterns. However, about 3% of the polypeptides examined were found to vary quantitatively among the eight subclones.

These results indicate that clonal cell lines can develop significant quantitative differences in the expression of polypeptides soon after they are separated from one another. The distribution of quantitatively variable spots among the different subclones indicated that there is a subpopulation of polypeptides, not necessarily specific to any of the subclones, whose rate of synthesis is variable.

Publications:

Huber, B. E., Heilman, C. A., Wirth, P. J., Miller, M. J. and Thorgeirsson, S. S.: Studies in gene transcription and translation in regenerating rat liver. Hepatology 6: 209-219, 1986.

Miller, M. J.: Quantitative analysis of two-dimensional gel electrophoretograms: Strategies and requirements for computerized analysis. In McEachron, D. L. (Ed.): Functional Mapping and Computer Analysis of Autoradiograms. Germany, Karger (In Press).

Miller, M. J.: Strategies and tools for the analysis of experiments involving multiple two-dimensional gel electrophoretograms. In Dunn, M. (Ed.): Electrophoresis '86. VCH Verlagsgesellschaft (In Press).

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05283-04 LEC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Transmission of Mammalian Genes with Expression-regulated Retrovirus Vectors		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: Michael G. Cordingley Others: Diana S. Berard Gordon L. Hager	Visiting Associate Microbiologist Head, Hormone Action & Oncogenesis Section	LEC NCI LEC NCI LEC NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Experimental Carcinogenesis		
SECTION Hormone Action and Oncogenesis Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 1.2	PROFESSIONAL: 0.7	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> Retrovirus-based vectors are ideally suited for the introduction of genetic information into mammalian cells, both in culture and in vivo. If expression of genes introduced with these vectors could be made conditional, their usefulness would be considerably enhanced. Regulation of expression from a glucocorticoid-induced promoter would be an attractive candidate, since most cell types contain functional glucocorticoid receptors. We have shown previously that expression of the v-Ha-ras oncogene can be made conditional when driven from the glucocorticoid responsive mouse mammary tumor virus promoter and that the transformed state of cells transfected with these fusions also is dependent on the presence of hormone. A preliminary series of vectors have been constructed with the MMTV v-Ha-ras cassette embedded in a retroviral backbone based on replication-competent murine leukemia virus, both in the parallel and anti-parallel transcriptional orientation. The neomycin resistance gene driven from the MuLV promoter has been included to provide selection independent of ras transformation. We find that hormone-dependent expression of the ras oncogene is observed with both orientations of the MMTV v-Ha-ras cassette. These experiments show, in principle, that a retrovirus-based vector can be developed which permits introduction of a given gene into cells with selection expression independent of the gene, and with subsequent expression of the gene subject to glucocorticoid regulation. </p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Michael G. Cordingley	Visiting Associate	LEC NCI
Diana S. Berard	Microbiologist	LEC NCI
Gordon L. Hager	Head, Hormone Action & Oncogenesis Section	LEC NCI

Objectives:

Construction and characterization of retrovirus-based vectors that permit the efficient introduction of genes into animal cells, both in culture and in vivo, in configurations such that expression of the genes are subject to regulation by glucocorticoid hormone.

The introduction of the ras oncogene, and other candidate oncogenes, into a variety of cell types with these vectors and subsequent characterization of oncogene activities based upon conditional expression of the oncogene product.

Methods Employed:

Functional elements involved in the replication, transmission and integration of retrovirus-based vectors are molecularly cloned using plasmid and bacteriophage vectors.

Ecotropic and amphotropic classes of the MuLV family of murine retroviruses are utilized as the transmission base for construction of vectors.

The v-Ha-ras oncogene is utilized as a test gene for functional expression of oncogenic activity in mouse cells after vector transmission. The neomycin resistance gene is utilized for dominant selection of vector transmission.

Functional expression of the ras gene product is monitored by morphological transformation of susceptible cells. Quantitative levels of ras expression are monitored by immunoprecipitation with monoclonal antibody of ras gene product or by S1 nuclease analysis of ras transcripts.

Vector DNA intermediates and integrated structures are characterized by restriction endonuclease analysis and Southern transfer blotting.

Vector RNA genomes and transcripts are characterized by S1 nuclease mapping and Northern transfer blotting.

Major Findings:

The structure and mode of replication of retroviruses makes them ideally suited for utilization in gene transfer experiments. In fact, retroviruses are natural vectors for introducing foreign DNA into vertebrate cell genomes. In their evolution acutely transforming retroviruses appear to have risen by incorporation of cellular DNA into the genome of weakly transforming retroviruses. There are now many examples of construction of replication-defective retrovirus vectors which

can stably transduce a foreign dominant selectable marker gene and replicate in the presence of helper, nondefective virus. Such viruses can infect susceptible cells efficiently and the selectable marker gene is stably incorporated in the cell genome in one to a few proviral copies. We have constructed a series of Moloney murine sarcoma viruses derived from retrovirus vectors designed to assess their potential for transduction of both a dominant selectable marker gene and a second nonselectable gene under independent transcriptional regulation. Initially a retrovirus vector (pCN10) was constructed which carried only the bacterial neomycin resistance gene (neo) from Tn5. Expression of this prokaryotic gene in eukaryotic cells renders them resistant to the drug G418. Analysis of the cell supernatant of pCN10-transfected helper cells revealed high titers of virus which conferred G418 resistance to NIH 3T3 cells. Cells which acquired G418 resistance carried a single copy of the drug resistance gene flanked by viral long terminal repeats, i.e., in the form of an integrated provirus. This virus vector was modified to incorporate a second gene, the coding sequences of the v-ras^H gene of Harvey murine sarcoma virus fused to the glucocorticoid inducible LTR of mouse mammary tumor virus. Four alternative configurations of this retrovirus vector were constructed (the pRNR-series). We have analyzed, in detail, two of these vectors (pRNR-6 and pRNR-16) for their ability to stably cotransmit both marker genes to infected cells and also the ability of the MMTV LTR-ras gene to respond to hormone stimulation after introduction into NIH 3T3 cells by infection with these vectors. We have analyzed both the number and the structure of the proviruses acquired by infectant cell lines by Southern blot analysis of infectant cell DNA with ras or neo gene probes. Northern blot analysis of ras-containing transcripts and nuclease S1 analysis of transcripts initiated at the MMTV promoter in a selection of infectant cell lines has revealed that the promoter can retain its ability to be regulated when resident within an integrated provirus. In addition, studies on transformed pRNR-6 infectant cell lines revealed a novel phenomenon. Selection for expression of the cotransduced neo gene in these cells was inefficient but when neo gene expression was attained, it resulted in switching of the cells to a nontransformed phenotype. Clearly the two independent transcription units within a single provirus are interacting, since expression of the neo gene appears to prohibit sufficient expression of the v-ras gene product for transformation.

Publications:

Cordingley, M., Richard-Foy, H., Lichtler, A., Ostrowski, M. C. and Hager, G. L.: The hormone response element of the MMTV LTR: A complex regulatory region. In Thompson, E. B. and Papaconstantinou, J. (Eds.): DNA: Protein Interactions and Gene Regulations, Galveston, The University of Texas Press (In Press).

Slagle, B. L., Wheeler, D. A., Hager, G. L., Medina, D. and Butel, J. S.: Molecular basis of altered mouse mammary tumor virus expression in the D-2 hyperplastic alveolar nodule line of Balb/C mice. Virology 143: 1-15, 1985.

Weeks, M. O., Hager, G. L., Lowe, R. and Scolnick, E. M.: Development and analysis of a transformation-defective mutant of Harvey murine sarcoma tk virus and its gene product. J. Virol. 54: 586-597, 1985.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05313-04 LEC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Early Events in Chemically Induced Rat Hepatocarcinogenesis		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Peter J. Wirth	Expert LEC NCI
Others:	Ritva P. Evarts	Veterinary Medical Officer LEC NCI
	Timothy Benjamin	Chemist LEC NCI
	Brian Huber	Senior Staff Fellow LEC NCI
	Dolores M. Schwartz	Biologist LEC NCI
	Snorri S. Thorgeirsson	Chief LEC NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Experimental Carcinogenesis		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.7	0.7	1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The project was initiated to study the sequence of events during chemically induced neoplasia using the rodent hepatoma model in combination with quantitative two-dimensional gel electrophoresis (2D-PAGE). Results obtained to date include: (1) analysis of polypeptide differences between normal rat liver and preneoplastic and neoplastic nodules generated using either the Solt-Farber regimen ("resistant hepatocyte" model) or via feeding the nongenotoxic inducer of peroxisome proliferation, ciprofibrate, revealed few qualitative but numerous quantitative polypeptide differences; (2) polypeptide expression in individual preneoplastic and neoplastic nodules induced via the Solt-Farber protocol or with ciprofibrate was very similar, suggesting a marked "homogeneity" rather than "heterogeneity" of polypeptide expression among the early lesions; (3) all Solt-Farber generated nodules stained strongly both for gamma-glutamyltranspeptidase (GGT) activity and for the placental form of glutathione-S-transferase (GST-P), while in the ciprofibrate induced nodules the expression of GGT and GST-P was not significantly different than control liver levels; (4) four qualitative polypeptide differences were noted in Solt-Farber-induced nodules as compared to normal liver. Two of these, polypeptides B (pI 6.25/41 kDa) and C (pI 6.75/24 kDa) were expressed in both ciprofibrate-induced and Solt-Farber-generated preneoplastic and neoplastic nodules. In addition five membrane-associated (5.25/59 kDa, 5.30/33 kDa, 5.25/27 kDa, 6.82/23.5 kDa, and 6.75/21 kDa) and four cytosolic polypeptides (6.20/45 kDa, 5.85/36 kDa, 5.05/34 kDa, 6.00/24 kDa) were coordinately expressed in both preneoplastic and neoplastic nodules from both ciprofibrate and Solt-Farber hepatocarcinogenesis models.</p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Peter J. Wirth	Expert	LEC NCI
Ritva P. Evarts	Veterinary Medical Officer	LEC NCI
Timothy Benjamin	Chemist	LEC NCI
Brian Huber	Senior Staff Fellow	LEC NCI
Dolores M. Schwartz	Biologist	LEC NCI
Snorri S. Thorgeirsson	Chief	LEC NCI

Objectives:

The process of chemical carcinogenesis has traditionally been divided into three phases: initiation, promotion and progression. Although the mouse skin was the first experimental model used to delineate these phases, the rodent liver is another tissue in which the initiation and promotion stages of chemical carcinogenesis can be clearly observed in vivo. The initiation events are generally believed to be the covalent interaction of a carcinogen with critical cellular macromolecules (e.g., DNA, RNA and proteins) in the target tissues which results in alteration of the normal functions within the cell. These alterations may result in changes in both gene expression and phenotypic characteristics and under appropriate conditions, namely, following promotion, develop into malignant tumor cells. Little is known concerning either the early cellular events in the initiation process or the gene products that control and maintain the initiated cells or how these products may change during hepatocarcinogenesis. Using the Solt-Farber resistant hepatocyte) and the Reddy (peroxisome proliferator-induced) initiation-promotion hepatocarcinogenesis protocols, studies have focused on the isolation and characterization (histologically, histochemically, and biochemically) of early preneoplastic, preneoplastic and neoplastic hepatocyte populations and examination of the differences in polypeptide expression in these populations using computer-assisted qualitative and quantitative two-dimensional gel electrophoresis (2D-PAGE) of total cellular proteins.

Methods Employed:

The principal methods employed are: (1) tissue culture techniques, (2) cell separation techniques--elutriation centrifugation, (3) histochemical staining, (4) enzyme assays involving radiometric (tritium, carbon-14, sulfur-35, phosphorus-32, and iodine-125) assays, (5) chemical and radiochemical synthesis, (6) differential centrifugation and chromatographic techniques, (7) autoradiography and fluorography; (8) two-dimensional gel electrophoresis, (9) Western transfer and immunoblot analysis, and (10) computer assisted quantitation of autoradiograms and silver stained gels.

Major Findings:

We have previously shown that 2D-PAGE can be used to reliably detect and quantitate changes in known polypeptide markers during hepatocarcinogenesis [e.g., gamma-glutamyltranspeptidase (GGT), glutathione-S-transferase (GST), DT-diaphorase, etc.]. Utilizing the Solt-Farber initiation-promotion protocol

for the generation of both preneoplastic and neoplastic liver nodule populations, we have identified one cytosolic (A, pI 6.8/57 kDa) and three membrane-associated polypeptides (B, 6.25/41 kDa; C, 6.75/26 kDa; D, 6.05/21 kDa) that are closely associated with early stages of hepatocarcinogenesis. These studies have now been extended to include polypeptide difference observed in hyperplastic nodules induced using the nongenotoxic peroxisome proliferator, ciprofibrate, and these changes have been compared to those observed using the Solt-Farber (SF) regimen. A large population of well-defined hyperplastic nodules were formed in male Fischer rats after feeding ciprofibrate (CP) (0.025%) for 9 months. Individual nodules (~20) were removed, small sections taken for histological evaluation and the remainder fractionated into cytosolic and crude membrane preparations using differential centrifugation. Histochemically, all the SF-induced nodules stained strongly for GGT activity, while none of the CP nodules was positive for GGT activity. Approximately 1000 polypeptides (silver stained) were analyzed on each electrophoretogram following 2D-PAGE over the pH range 5.0-7.4 and molecular weight range 15-120 kDa. A marked homogeneity of polypeptide expression, similar to that seen in the SF nodules, was observed among the individual preneoplastic and neoplastic CP-induced nodules. Quantitative comparison of polypeptides within the same tissue type (i.e., preneoplastic vs preneoplastic and neoplastic vs. neoplastic) revealed a very tight quantitative correspondence between paired polypeptide spots in both cytosolic and membrane fractions with matching correlation coefficients (CE) in the range of 0.90-0.96 (CE = 1.00 if all spots identical). Comparison of cytosolic and membrane-associated polypeptides from normal liver with either preneoplastic (CE = 0.80 and 0.78) or neoplastic nodules (CE 0.76 and 0.70) or comparison of polypeptides from preneoplastic nodules with neoplastic nodules (CE 0.90 and 0.91) showed a greater scattering of polypeptides, indicating a greater modulation of polypeptide synthesis during hepatocarcinogenesis. Comparison of 700-800 cytosolic and membrane-associated polypeptides from preneoplastic and neoplastic nodules revealed that roughly 3-9% of the membrane and 2-4% of the cytosolic polypeptides were undergoing quantitative changes of at least fourfold during hepatocarcinogenesis.

Although there was a marked similarity of polypeptide expression among the individual CP-induced nodules, comparison of polypeptide expression in CP-induced nodules with that in SF nodules revealed a few qualitative but numerous quantitative polypeptide differences. In particular, in SF nodules the expression of dT-diaphorase (expressed as percent of total integrated polypeptide density on each gel) is increased two- to threefold (90.30 ± 0.05 vs 0.13 ± 0.04), while the Yp subunit of GST-P is increased 20- to 30-fold (1.45 ± 0.15) as compared to normal liver (0.03 ± 0.01). In CP induced nodules DT-diaphorase and the Yp subunit of GST-P are not increased over normal levels. Furthermore, polypeptides A (6.80/57 kDa) and D (6.05/21 kDa) are not expressed in CP-induced nodules; however, B (6.25/41 kDa) and C (6.75/24 kDa) are expressed in both CP- and SF-induced preneoplastic and neoplastic nodules. In addition, five membrane-associated (5.25/59 kDa, 5.30/33 kDa, 5.25/27 kDa, 6.82/23.5 kDa, and 6.75/21 kDa) and four cytosolic polypeptides (6.20/45 kDa, 5.85/36 kDa, 5.05/34 kDa, and 6.00/24 kDa) were coordinately expressed in both preneoplastic and neoplastic nodules from both CP and SF hepatocarcinogenesis models.

Publications:

Wirth, P. J., Benjamin, T., Schwartz, D. M., and Thorgeirsson, S. S.: Sequential analysis of chemically induced hepatoma development in rats by two-dimensional electrophoresis. Cancer Res. 46: 400-413, 1986.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05315-04 LEC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Cell Surface Proteins and Cellular Adhesion in Hepatocarcinogenesis		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Snorri S. Thorgeirsson	Chief LEC NCI
Others:	Hannu Raunio	Visiting Fellow LEC NCI
	Ritva P. Evarts	Veterinary Medical Officer LEC NCI
	Ryuichi Konno	Visiting Fellow LEC NCI
	Peter J. Wirth	Expert LEC NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Experimental Carcinogenesis		
SECTION Chemical Carcinogenesis Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.3	0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> The main objective of this project is to analyze changes in homotypic cell-to-cell adhesion during the evolution of chemically induced rat hepatocarcinogenesis and to identify the cell surface proteins that are involved in this process. Examination of intercellular homotypic adhesive properties of 14 clones derived from a neonatal Fischer rat liver epithelial cell line (FNRL) showed that the clones differ both in response to dissociation by trypsin treatment and reaggregation rates. Increased adhesion among the clones was associated with an increased proportion of aneuploid cells in the clones. The parent cell line and the clones were unable to grow in soft agarose in the absence or presence of 2 ng/ml of epidermal growth factor (EGF). The rat hepatoma cell line H4-II-E showed negligible capacity to reaggregate after dissociation into single cells and these cells readily formed colonies in soft agarose. Markedly elevated amounts of two acidic glycoproteins (105 kd and 67 kd) were detected in the "most adhesive" clone when the two-dimensional gel electrophoresis (2D-PAGE) pattern of concanavalin A (Con A)-binding glycoproteins in this clone was compared to that of the "least adhesive clone." 2D-PAGE patterns of plasma membrane glycoproteins isolated by Con A affinity chromatography from normal, preneoplastic and neoplastic livers showed both qualitative and quantitative changes among the samples. Qualitative differences consisted of four new polypeptides appearing in preneoplastic liver versus control liver, four polypeptides lacking in neoplastic liver, and five polypeptides appearing new in neoplastic liver compared with control liver. These findings support the hypothesis that modulation of normal cell surface components, especially glycoproteins involved in cell-to-cell or cell-to-matrix adhesion and communication, may be responsible for some of the biological behavior of cancer cells. </p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Snorri S. Thorgeirsson	Chief	LEC NCI
Hannu Raunio	Visiting Fellow	LEC NCI
Ritva P. Evarts	Veterinary Medical Officer	LEC NCI
Ryichi Konno	Visiting Fellow	LEC NCI
Peter J. Wirth	Expert	LEC NCI

Objectives:

The main objectives of this project are: (1) to analyze changes in homotypic cell-to-cell adhesion during different phases of chemically induced rat hepatocarcinogenesis; to identify and characterize, primarily by two-dimensional gel electrophoresis, the cell surface proteins that are involved in the cell-to-cell adhesion; and to compare these proteins in normal, preneoplastic and neoplastic cells. Our aim is to identify alterations in cell surface protein patterns that distinguish the malignant cell from the normal cell and to examine the regulation of those surface proteins that are highly associated with the neoplastic process. (2) To characterize the changes in cell-to-cell adhesion caused by transforming growth factors.

Methods Employed:

- (1) In vitro culturing of liver-derived epithelial cells and rat kidney cells.
- (2) In situ perfusion technique to obtain viable hepatocytes from rat liver.
- (3) Cell-to-cell adhesion assay using an electronic particle counter.
- (4) Chromosome analysis using the conventional colcemid-trypsin treatment.
- (5) The soft agarose method to delineate anchorage-independent growth of cells.
- (6) Analysis of cellular glycoproteins by first selecting the proteins by lectin affinity chromatography and then applying two-dimensional gel electrophoresis to separate the proteins. (7) Isolation of plasma membrane fractions of livers by using a series of isopycnic and differential centrifugations and one-step sucrose gradient centrifugation.

Major Findings:1. Polypeptide changes in a subset of membrane-associated glycoproteins.

Because of the large number of membrane-associated proteins and the initial finding that very few changes can be detected between normal, preneoplastic and fully neoplastic tissue, we felt it would be helpful to select a subset of membrane-associated proteins where changes could be detected without interference from the bulk of proteins which does not change during the neoplastic process. This was accomplished by using Con A affinity chromatography to specifically select for the glycoproteins present in the membrane protein mixture which have affinity to this lectin. When the fraction specifically bound to Con A was separated by 2D-PAGE, the number of spots detected was approximately 300.

The polypeptide pattern obtained after selection for Con A-binding glycoproteins in the plasma membrane was highly reproducible within the three groups under investigation. As with the nonselected proteins, the correlation coefficients for intragroup comparisons are very high: 0.94 for two gels from normal liver and 0.95 ± 0.01 for three gels from different preneoplastic livers. An exception was the neoplastic group. The three liver adenocarcinomas compared yielded a correlation coefficient of 0.81 ± 0.12 , reflecting a more pronounced heterogeneity among the tumor samples compared with normal and preneoplastic liver. The same pattern was observed when the groups were compared with each other; a high correlation between control and preneoplastic liver plasma membrane-associated glycoproteins and a wider scattering when normal and neoplastic and preneoplastic and neoplastic liver were compared (correlation coefficients 0.80 ± 0.06 and 0.77 ± 0.5 , respectively). This scattering is also reflected in the number of major proteins (0.05% cut-off) changing more than fourfold: 1.0% of polypeptides show a more than fourfold change when control liver was compared with preneoplastic liver, whereas 11.8% of polypeptides change more than fourfold when control samples were compared with neoplastic ones.

The changes between normal and preneoplastic tissue were small with the exception of six proteins. Four new proteins emerged in the gels from preneoplastic liver plasma membrane preparations, and two other polypeptides were markedly increased in the preneoplastic liver. In contrast, comparison between control and fully neoplastic tissue revealed several changes.

Four de novo proteins were detected in preneoplastic versus control liver, whereas no proteins were missing in the gels from preneoplastic liver. Gels from fully neoplastic liver consistently lacked four proteins, and five proteins emerged new in neoplastic liver compared with control liver. One of the de novo proteins was common to both preneoplastic and neoplastic livers, whereas the others were different.

2. Adhesive properties of liver-derived epithelial cell in culture.

Transformation of cells is associated with numerous changes in the biological behavior of the cells. For example, transformed cells are able to grow in semi-solid medium (anchorage-independent growth), and they show decreased adhesion to surfaces such as glass or plastic. Changes in homotypic cell-to-cell adhesion also occur after transformation, but numerous studies have not yielded a coherent picture on whether transformed cells show reduced or increased homotypic adhesiveness. It is also unclear how these changes in cell-to-cell adhesion are reflected in the biology of tumor cells.

To relate changes of liver-derived epithelial cell adhesion characteristics to phenomena associated with malignancy, we studied several individual clones established from a Fischer rat liver-derived cell line (FNRL). The cell-to-cell adhesive properties, chromosome number and growth on soft agar was determined for 14 clones and the parent FNRL cell line at two different passage levels. The rat hepatoma line H4-II-E was used as a reference representing a transformed liver cell line. The homotypic cellular adhesive properties were determined by dissociating confluent monolayers of cells with trypsin and measuring the amount of aggregates greater than 30 microns in diameter with a Coulter Counter

immediately after dissociation and after various times of shaking the dissociated cells in the culture medium. Detachment of cells with proteolytic treatment yields a mixture of single cells and small aggregates. During 3 hours of shaking at 37°C, the number of aggregates greater than 30 micromoles in diameter increases steadily, accompanied by a parallel reduction in the amount of single cells.

The comparison of adhesive properties among the individual clones was accomplished by determining the ability of the clones to form 30 micromole aggregates within 3 hours of shaking. It was evident that the individual clones differ widely in both response to treatment with dissociating agents and in their reaggregating ability. The two extreme clones, 201 and 67, showed a 105-fold difference in the amount of aggregates over 30 microns immediately after detachment. The H4-II-E cells readily dissociated into single cells, and virtually no formation of aggregates greater than 30 microns was observed.

The modal chromosome number and the percentage of aneuploid cells was determined for each clone. Of the 14 clones tested, 11 had a normal modal number of 42. Of the three aneuploid clones, two belonged to the group which was highly adhesive as judged by the presence of aggregates after detaching the cells and after the 3 hour shaking period. The parent FNRL cell line had a normal modal chromosome number of 42. As expected, all H4-II-E cells showed an aneuploid chromosome number (modal = 54).

When plated in 0.3% agarose, the parent FNRL cell line and the clones derived from it were unable to grow in the presence or absence of EGF (2 ng/ml). In contrast, the H4-II-E cells formed large colonies within a week after plating in soft agar both in the presence and absence of EGF.

A subset of membrane-associated glycoproteins was selected by Con A affinity chromatography from the least adhesive clone 201, the most adhesive clone 67, and the H4-II-E cells. After selection of Con A-binding glycoproteins, the proteins were separated with high-resolution 2D-PAGE. The accuracy and reproducibility of the polypeptide separation was assessed by comparing three different preparations from each clone. The average correlation coefficient for intensities in matching spots was 0.90 for gels derived from clone 201, 0.94 for clone 67, and 0.97 for the H4-II-E cell line.

Publications:

None

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05317-03 LEC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Opal Suppressor tRNA in Human and Other Genomes		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Dolph L. Hatfield	Research Biologist LEC NCI
Others:	Bjeong Jae Lee	Visiting Fellow LEC NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Experimental Carcinogenesis		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> The only naturally occurring nonsense suppressor tRNAs described in higher eukaryotes are two opal suppressor serine tRNAs that occur in vertebrate tissues. These tRNAs have several unique features: (1) they are 90 nucleotides in length and thus are the longest tRNAs sequenced to date; (2) they are phosphorylated on their serine moiety to form phosphoseryl-tRNA; (3) they have few modified bases compared to other tRNAs; (4) they are encoded by a single gene even though several pyrimidine transitions occur post-transcriptionally and one of the transitions occurs in the anticodon; (5) the primary transcript arises, unlike any other known tRNAs, without processing on the 5' side of the gene product; and (6) the gene evolved more recently than other tRNA genes. An examination of the approximate origin and distribution of the opal phosphoserine gene in nature has shown that the gene occurs only in the Animal Kingdom and not in representatives of the Monera, Protist, Fungi and Plant Kingdoms. Among animals the gene occurs in members of the Phylum Chordata (tunicate, amphioxus, lamprey, hag fish, horned shark, winter flounder, Xenopus, chicken and bovine), but not in members within the Phyla Arthropoda, Mollusca, Aschelminthes or Porifera. The genes encoding the opal suppressor tRNAs which have been isolated and sequenced from human, rabbit, chicken and Xenopus genomes are transcribed in vivo in Xenopus oocytes and are transcribed in vitro in HeLa cell extracts. Fingerprints of the processed transcript from the Xenopus gene show that the gene is faithfully transcribed and that initiation of transcription occurs at the first nucleotide within the gene. </p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Dolph L. Hatfield	Research Biologist	LEC NCI
Bjeong Jae Lee	Visiting Fellow	LEC NCI

Objectives:

The major goals of the project are to understand the structure, expression, function and evolutionary origin of the opal suppressor tRNA genes and the role that the products of these genes have in protein phosphorylation.

Specific steps to achieve these goals are: (1) to isolate and characterize opal suppressor tRNA genes from genomes of higher eukaryotes; (2) to sequence the genes and their flanking DNA segments; (3) to investigate the structure of the genomic regions that contain these genes with respect to transcription and evolutionary conservation; (4) to study the control of transcription and using in vivo and in vitro transcription system; (5) to use in vivo transcription systems to study processing and localization of the tRNA product; (6) to make site-specific mutations in the internal control region and in the anticodon region of the tRNA genes and to replace the 5' flanking sequence with that of another tRNA gene in order better to understand better the expression and cellular function of these genes; (7) to subclone the opal suppressor tRNA gene which has the 5' flanking sequence replaced so that it will make product in high yields and subclone the ochre suppressor tRNA gene which has been generated by site-specific mutagenesis into a mammalian cell line in order to determine the effects of these suppressors on cellular function (i.e., if the gene products are phosphorylated on the serine moiety and if phosphoserine is incorporated directly into protein); and (8) to investigate the distribution and evolutionary origin of this unique gene in nature.

Methods Employed:

Genomic DNAs from a wide variety of organisms were obtained from a number of laboratories or were isolated by standard techniques of preparing DNA. These DNAs were digested with restriction enzymes and electrophoresed on agarose gels, Southern blotted and hybridized with an appropriate probe.

Transcription of the opal suppressor tRNA genes were carried out in the presence of extracts of HeLa cells which were used as a source of RNA polymerase III. Transcripts were digested with RNase, fingerprinted and the resulting oligonucleotides identified by standard techniques. The opal phosphoserine tRNA genes were also injected into *Xenopus* oocytes, the transcripts extracted and electrophoresed on polyacrylamide gels in order to examine their transcription in vivo.

Major Findings:

Four opal suppressor tRNA genes and two pseudogenes have previously been sequenced from human, rabbit, chicken, and *Xenopus* genes. The pseudogenes occur

in mammalian genomes and the gene appears to occur in single gene copy in each of these organisms. An examination of the distribution and evolutionary origin of the opal suppressor gene in nature shows that it is not present in genomic DNAs of representatives of the Monera (*Micrococcus*), Protist (*Ochromonas*, *L. major* and *Physarum*), Fungi (yeast) or Plant (tomato) Kingdoms. It is present in the genomic DNAs of representatives of the higher phyla of the Animal Kingdom, but not in representatives of the lower phyla. The gene occurs in the Phylum Chordata (tunicates, amphioxus, lamprey, hag fish, horned shark, winter flounder, *Xenopus*, chicken and bovine), but not in the Phyla Arthropoda (*Drosophila* and horse shoe crab), Mollusca (clam, oyster and snail), *Aschelminthes* (*Ascarus* and *C. elegans*) and Porifera (sponge). In addition, comparison of the sequences of the opal suppressor phosphoserine tRNAs to those of 478 other tRNAs by a computer program demonstrated that there is no evolutionary linkage between the opal suppressors and any known tRNA sequence. These observations demonstrate that the opal suppressor tRNAs evolved more recently and independently of other tRNA genes.

The genes from human, rabbit, chicken and *Xenopus* genomes are transcribed in *Xenopus* oocytes and, therefore, are transcribed in vivo. Furthermore, the human pseudogene is also transcribed in vivo. The fact that this pseudogene arose by combination with two Alu family members makes the determination of the cellular role of this unique transcript an important study. The human and *Xenopus* genes are transcribed in vitro in HeLa cell extracts. Fingerprints of the *Xenopus* transcripts show that the genes are faithfully transcribed and most importantly that initiation of transcription of these genes occurs on the first nucleotide within the gene.

Publications:

Smith, D. W. E. and Hatfield, D. L.: Effects of post-transcriptional base modifications on the site-specific function of transfer RNA in eukaryotic translation. J. Mol. Biol. 189: 663-671, 1986.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER 701CP05373-03 LEC
PERIOD COVERED <u>October 1, 1985 to September 30, 1986</u>		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <u>Purification of Rat Hepatic Proliferation Inhibitor</u>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: Henry C. Krutzsch	Expert	LEC NCI
Others: Snorri S. Thorgeirsson	Chief	LEC NCI
Anthony C. Huggett	Visiting Fellow	LEC NCI
Peter J. Wirth	Expert	LEC NCI
James B. McMahon	Expert	LETM NCI
COOPERATING UNITS (If any)		
<u>None</u>		
LAB/BRANCH <u>Laboratory of Experimental Carcinogenesis</u>		
SECTION <u>Biopolymer Chemistry Section</u>		
INSTITUTE AND LOCATION <u>NCI, NIH, Bethesda, Maryland 20892</u>		
TOTAL MAN-YEARS: <u>1.4</u>	PROFESSIONAL: <u>1.4</u>	OTHER: <u>0</u>
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> The goal of this project is to isolate and study the endogenous protein from rat liver that causes reversible, cytostatic inhibition of liver cell proliferation. Research efforts have been focused on purifying this hepatic proliferation inhibitor (HPI) so that a number of structural and biological studies can be carried out. The strategy previously developed for isolating HPI, involving extraction from liver, ammonium sulfate and ethanol precipitation, and chromatography by phenyl sepharose, gel filtration, and high resolution cation- and anion-exchange FPLC, was initially applied. HPI from this procedure was highly active, but not homogeneous. Work was directed at obtaining completely homogeneous preparations of HPI. The first approach involved further purification steps on the HPI obtained as described above. Weak anion- and cation-exchange and reverse phase HPLC, chromatography and hydrophobic interaction FPLC, and denaturing and nondenaturing one-dimensional electrophoresis were explored in this effort. In the second approach, a new set of chromatographic steps was substituted for the four column steps previously used. The new purification procedure involves chromatography by DEAE-cellulose, gel filtration and high resolution chromatofocusing and hydrophobic interaction FPLC. The HPI obtained by this means has about 100-1000 times the specific activity of HPI obtained by the earlier methods, with an estimated ED 50 in the range of 50-500 pg/ml. </p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Henry C. Krutzsch	Expert	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI
Anthony C. Huggett	Visiting Fellow	LEC	NCI
Peter J. Wirth	Expert	LEC	NCI
James B. McMahon	Expert	LETM	NCI

Objectives:

The objective of this project is to isolate and study the endogenous polypeptide in liver that inhibits hepatocyte proliferation, called hepatic proliferation inhibitor (HPI). The initial goal of this project is to develop a method of purification for obtaining sufficient amounts of HPI so that extensive characterization of this molecule can then be undertaken. Structural studies will allow antibody production, DNA cloning and comparison with other biologically active proteins.

Methods Employed:

For HPI isolation in this work, standard procedures for tissue homogenization, ammonium sulfate and ethanol precipitation, column chromatography and fast protein liquid chromatography (FPLC) conditions were utilized. The assay used to detect HPI activity in column fractions was the one previously developed in this laboratory that uses 96 well microtiter plates and determines inhibition of cellular proliferation by use of a viable fluorescent dye that measures the total cellular DNA present in each well.

Major Findings:

The methodology previously developed for isolating HPI, involving extraction from rat livers, ammonium sulfate and ethanol precipitation, and chromatography by phenyl sepharose, gel filtration and high resolution cation- and anion-exchange FPLC, was initially applied. HPI obtained from this procedure was highly active, but not homogeneous. Therefore, work was targeted at obtaining completely pure HPI using the material obtained after the purification scheme described above as the starting material. Weak anion- and cation-exchange reverse phase HPLC chromatofocusing and hydrophobic interaction FPLC and denaturing and nondenaturing one-dimensional gel electrophoresis were explored for this purpose. In this series of experiments, non-denaturing one-dimensional gel electrophoresis were explored for this purpose. In the second approach, a new series of chromatographic steps was substituted for the four column steps previously used. The new purification procedure derived from this work uses the same extraction and precipitation steps used in the previous procedure and involves chromatography by DEAE-cellulose, gel filtration, and high resolution chromatofocusing and hydrophobic interaction FPLC. The HPI obtained by this means has about 100-1000 times the specific activity of HPI obtained by the previous purification scheme, with an estimated ED 50 in the range of 50-500 pg/ml. From the chromatofocusing chromatography, the pI of HPI appears to be about 5.5. Recent work has been focused on using

this new purification methodology to obtain further quantities of HPI, so that SDS-PAGE analysis for purity can be carried out, and so that the production of monoclonal antibodies can be initiated.

Publications:

Cheng, K. C., Krutzsch, H. C., Grantham, P. H., Park, S. S., Gelboin, H. and Friedman, F.: Amino-terminal sequence and structure of monoclonal antibody immunopurified cytochromes P-450. Biochemistry 25: 2397-2402, 1986.

Deibler, G. E., Krutzsch, H. C. and Kies, M. W.: A new form of myelin basic protein found in human central myelin. J. Neurochem. (In Press).

Friedman, F. K., Robinson, R. C., Krutzsch, H. C., Grantham, P. H., Park S. S. and Gelboin, H. V.: Monoclonal antibody directed isolation and amino-terminal sequence analysis of phenobarbital induced rat liver cytochromes P-450. Biochem. Biophys. Res. Commun. 129: 926-933, 1985.

Krutzsch, H. C.: Polypeptide sequence analysis using gas chromatography-mass spectroscopy. In Shively, J. E. (Ed.): Methods of Protein Microcharacterization, Chapter 15, Clifton, N. J., Humana Press, 1986, pp. 381-481.

Krutzsch, H. C.: Protein/peptide sequence analysis by mass spectrometry. In Bhowm, A. S. (Ed.): Protein/Peptide Sequence Analysis: Current Methodologies, Cleveland, Ohio, CRC Press (In Press).

Law, M. J., Deibler, G. E., Martenson, R. E. and Krutzsch, H. C.: Plasmin-sensitive bonds in rabbit myelin basic protein. J. Neurochem. 45: 1232-1243, 1985.

Richards, W. L., Song, M.-K., Krutzsch, H. C., Evarts, R. P., Marsden, E. and Thorgeirsson, S. S.: Measurement of cell proliferation in microculture using Hoechst 33342 for the rapid semi-automated microfluorometric determination of chromatin DNA. Exp. Cell Res. 159: 235-246, 1985.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05374-03 LEC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Structural and Physicochemical Studies of Proteins Relevant to Tumorigenesis		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Peter P. Roller	Head, Biopolymer Chemistry Section LEC NCI
Others:	Snorri S. Thorgeirsson	Chief LEC NCI
	Chien-Hua Niu	Expert LEC NCI
	Preston H. Grantham	Chemist LEC NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Experimental Carcinogenesis		
SECTION Biopolymer Chemistry Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.6	0.8	0.8
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> This project involves studies on the chemical structure and physicochemical characteristics of certain natural biopolymeric materials and their synthetic analogs with the aim of relating the resulting structural information to their biological mode of action. Current emphasis is focused on proteins that play a role in cell growth regulation, cell transformation or differentiation. Emphasis is placed in applying the modern methods of mass spectrometry, chromatography and of chemical methods of sequencing. Projects include: (1) Development of methods and application of fast atom bombardment mass spectrometry. Systematic investigation of 9 N-terminal pyroglutamate-blocked peptides demonstrated structurally characteristic spectral features, and also indicated that C and Z type of amino acid sequence-determining ions predominate in the spectra. Bromine-containing group-specific labeling reagents were found to be useful in enhancing the sequence specific ions in the spectra in general. Spectral measurements on several synthetic transforming growth factor-alpha polypeptide segments secured the monomeric/oligomeric status and the oxidation state of the thiol groups present in the molecules. (2) Structural studies on gamma-glutamyl transpeptidase, a hepatic tumor marker enzyme. Amino acid sequencing studies and two-dimensional gel electrophoretic studies have been completed to the extent that the sequence information could be compared to recent DNA cloning data by others. Our electrophoretic isozyme analysis results offer a method of general applicability to inter-tissue and inter-species comparative studies. </p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Peter P. Roller	Head, Biopolymer Chemistry Section	LEC NCI
Snorri S. Thorgeirsson	Chief	LEC NCI
Chien-Hua Niu	Expert	LEC NCI
Preston H. Grantham	Chemist	LEC NCI

Objectives:

To study, in some detail, the chemical structure and physicochemical characteristics of certain natural biopolymeric materials, with emphasis on proteins that play a role in cell growth regulation such as enzymes, hormones, growth factors and tumor indicator proteins. To develop and apply modern mass spectrometric methods to the solution of structural problems, particularly to protein sequencing.

Methods Employed:

(1) Fast atom bombardment negative and position ion mass spectrometry; (2) proton and carbon-13 nuclear magnetic resonance spectroscopy on samples in the solution state; (3) circular dichroism spectropolarimetry; (4) spectrophotometry; (5) chemical modification and derivatization of peptides and proteins; (6) high pressure liquid chromatography, gel filtration, partition and affinity chromatographies; (7) two-dimensional gel electrophoresis; (8) Edman sequencing of proteins, and (9) amino acid analysis.

Major Findings:

Peptide sequencing by fast atom mass spectrometry (FAB). There are a number of protein structural problems, where the method of FAB mass spectrometry ideally complements the classical Edman chemistry-based sequencing information. The method yields accurate molecular weight information on protein fragments up to a mass of 4000 on the instrument available in our laboratory. Amino acid sequence information can be obtained equally well on peptides that are N-terminally blocked or that contain modified amino acids or have conjugating functional groups attached. The method is suited for confirmation or correction of sequence determined by other methods, including DNA cloning.

A large number of peptides and proteins are N-terminally blocked. N-Terminal sequencing of these by the Edman method poses special problems. We examined, in detail, the spectra of a set of nine synthetic N-terminal pyroglutamate-containing peptides to explore the presence of characteristic features that may prove to be useful in providing structural information on unknowns. The spectra consistently showed the presence of immonium ions in the low mass range indicative of the amino acid components. The presence of pyroglutamate is indicated by a significant ion at m/z 84 in the spectra. Several of the peptides fragment in a consistent fashion giving only either C⁺ or Z⁺ type sequence ions, while others give a variety of ion types. Insight into the fragmentation pattern of this class of peptides allows for a better predictive ability when sequencing unknown substances.

The detection limit of the FAB mass spectrometric method is 10 to 50 times better for the determination of molecular weight-determining ions, than for amino acid sequence determining ions. In order to increase the sequence information obtainable from the spectra, we have reported in the past on the utility of labeling peptides on specific amino acids with bromine containing reagents. It is found that the bromine atom partially back-exchanges with the FAB liquid matrix during the measurement, especially when the bromine is present on an aromatic amino acid residue, such as is the case with (3,5-dibromotyrosine)-leu-enkephaline. To prevent this complicating factor in the analysis, we are exploring the use of radical scavenger matrices, such as 3-nitrobenzyl alcohol.

We have made FAB mass spectral measurements on two synthetic transforming growth factors-alpha segment peptides, prepared in our laboratory, to evaluate their oxidation state and monomeric/oligomeric status. The desired 12-residue cyclic disulfide was satisfactorily characterized by giving an isotopic molecular weight of 1405.65 (error: 0.20 amu), and analogously for the 17-mer a chemical molecular weight of 1919.50 (error: 0.32 amu). To obtain sequence information on cyclic peptides, experiments are in progress to reductively cleave the peptide followed by alkylation of the thiol groups with 4-vinylpyridine. The resulting linear peptides will be subjected to further spectral measurements.

Publications:

Aszalos, A., Bax, A., Burlinson, N., Roller, P. and McNeal, C.: Physico-chemical and microbiological comparison of nystatin, amphotericin A and amphotericin B, and structure of amphotericin A. J. Antibiotics 38: 1699-1713, 1985.

Cone, J.L., Glowinski, I. B., Wirth, P. J., Grantham, P. H. and Roller, P. P.: Structural studies and two-dimensional gel electrophoresis of gamma-glutamyl transpeptidase. Arch. Biochem. Biophys. 247: 165-170, 1986.

Fox, C. H., Johnson, F. B., Whiting, J. and Roller, P. P.: Formaldehyde fixation. J. Histochem. Cytochem. 33: 845-853, 1985.

Vu, V. T., Grantham, P. H., Roller, P. P., Hankins, w. D., Wirth, P. J. and Thorgeirsson, S. S.: Formation of DNA adducts from N-acetoxy-2-acetylamino-fluorene and N-hydroxy-2-acetylaminofluorene in rat hemopoietic tissue in vivo. Cancer Res. 46: 233-238, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05379-03 LEC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <u>Analysis of Polypeptide Changes during Cellular Differentiation and Transformation</u>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: Peter J. Wirth	Expert	LEC NCI
Others: Stuart H. Yuspa		
Henry Hennings	Chief	LCCTP NCI
Curtis Harris	Expert	LCCTP NCI
Brenda I. Gerwin	Chief	LHC NCI
Joseph A. DiPaolo	Expert	LHC NCI
Jay Doniger	Chief	LB NCI
Jay Doniger	Expert	LB NCI
COOPERATING UNITS (if any)		
None		
LAB/BRANCH Laboratory of Experimental Carcinogenesis		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 0.4	PROFESSIONAL: 0.4	OTHER: 0
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither		
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p> This project was initiated to analyze, both qualitatively and quantitatively, changes in total cellular protein patterns during cellular differentiation and transformation using the technique of quantitative two-dimensional electrophoresis. A cell culture system has been developed for the study of differentiation of primary normal human bronchial epithelial (NHBE) cells by 12-0-tetradecanoyl-13-acetate (TPA) or transforming growth factor type beta (TGF-beta). Two-dimensional gel electrophoresis (2D-PAGE) of [¹⁴C]-labeled polypeptides following treatment of NHBE cells and the human lung carcinoma cell line, A1146, with TPA and TGF-beta resulted in only quantitative polypeptide differences. NHBE cells showed a more pronounced modulation of polypeptide expression following treatment with TPA (10 nM) and TGF-beta (30 pg/ml) than A1146 cells, a cell line refractory to differentiation induction by TPA and TGF-beta. After 24 hr treatment of NHBE cells with TPA and TGF-beta, 15 polypeptides were specifically modulated by TPA, 4 were modulated by TGF-beta alone, and 6 were affected by both TPA and TGF-beta. Prolonged exposure (72 hr) resulted in predominately (38/42) TPA-induced changes and only one polypeptide was specifically modulated by TGF-beta. Of the 42 polypeptides significantly modulated at 72 hr, 4 polypeptides (5.85/88 kDa; 6.10/40 kDa; 5.00/40kDa; and 5.80/28 kDa) also exhibited significant quantitative modulation after 24 hr treatment. </p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Peter J. Wirth	Expert	LEC	NCI
Stuart H. Yuspa	Chief	LCCTP	NCI
Henry Hennings	Expert	LCCTP	NCI
Curtis Harris	Chief	LH	NCI
Brenda I. Gerwin	Expert	LH	NCI
Joseph A. DiPaolo	Chief	LB	NCI
Jay Doniger	Expert	LB	NCI
Timothy Benjamin	Chemist	LEC	NCI
Dolores M. Schwartz	Biologist	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI

Objectives:

The overall objective of this project is to employ the computer-based two-dimensional electrophoresis (2D-PAGE) of total cellular polypeptides to analyze, both qualitatively and quantitatively, the changes in the polypeptide patterns during cellular transformation and differentiation. We plan to use this experimental technique to critically examine the hypothesis that neoplasia results from a "block" in normal cellular differentiation. The experimental systems that we are currently examining include cultured mouse epidermal cells (keratinocytes), human bronchial epithelial cells, and Syrian hamster fetal cells (HFC) in the study of blocked differentiation versus neoplastic transformation.

Methods Employed:

The principle methods employed are: (1) tissue culture techniques; (2) histochemical staining; (3) differential centrifugation; (4) autoradiography and fluorography; (5) two-dimensional electrophoresis; (6) computer-assisted quantitation of autoradiograms and silver stained gels.

Major Findings:

Normal human bronchial epithelial cells (NHBE) can be induced to undergo terminal squamous differentiation (including an increase in cell surface area, formation of cross-linked envelopes, and cessation of cell division) in the presence of TPA (3 nM) or certain blood serum-derived (BDS) growth factors. In contrast, however, various lung carcinoma cell lines and Harvey v-ras DNA transfected NHBE (TBE-1 cells) are resistant to induction of terminal squamous differentiation by either BDS or 100 nM TPA. These results and the work of others suggest that an aberrant control of normal differentiation is positively correlated with malignant transformation (i.e., malignant cells have a reduced capacity to respond to factors which induce terminal differentiation in normal cells).

NHBE, TBE-1 and A1146 (lung carcinoma cell line) were exposed to either 10 nM TPA or TGF-beta (30 pg/ml) for 24 and 72 hr in LHC-8 medium and labeled for 24 hr with [¹⁴C]-amino acids. Although LHC-8 medium has been shown to be the optimum culture medium for differentiation studies utilizing TGF-beta, TBE-1 cells failed to grow

in this medium and, therefore, did not incorporate sufficient [^{14}C] to allow for 2D-PAGE analysis of NHBE; A1146 cells adapted quite well to LHC-8 medium and were labeled with a high incorporation of radioactive amino acids. We are currently in the process of adapting TBE-1 cells to growth in LHC-8 medium for future 2D-PAGE analysis.

Since NHBE and A1146 cells adapted quite well to LHC-8 medium, the effects of TPA and TGF-beta-induced differentiation in these two cell lines were studied. Following 2D-PAGE separation of total cellular polypeptides from untreated NHBE and A1146 cells, numerous qualitative and quantitative polypeptide differences were noted between the two cell lines. Approximately 1000-3000 polypeptides were readily detected on each autoradiogram over the pI range of 4.5-7.5 and molecular weight range of 15-130 kDa. Treatment of NHBE and A1146 cells with either TPA or TGF-beta for 24 or 72 hr failed to induce the synthesis of any qualitatively new polypeptides in either line. TPA and TGF-beta treatment did, however, result in the quantitative modulation of numerous polypeptides, especially in the responsive NHBE cells. The pattern of polypeptide expression in A1146 cells 24 hr after treatment with either TPA or TGF-beta was compared with those from untreated A1146 cells. Neither TPA nor TGF-beta had any effect on polypeptide expression. TPA and TGF-beta affected the synthesis of 61 (6.3% of the total number of polypeptides compared) and 73 (8.0%) polypeptides (>900 compared), respectively, by two-fold or greater. This was not, however, significantly different from comparison of two untreated control samples (59/1046; 5.7%). Similar effects were observed after 72 hr treatment. The expression of polypeptides from NHBE cells, on the other hand, were significantly affected by both TPA and TGF-beta. Roughly 11% (76/717) and 13% (94/725) of the polypeptides in NHBE cells underwent quantitative changes of at least twofold following 24 hr treatment with TGF-beta and TPA, respectively. Treatment of NHBE cells for 72 hr did not significantly alter the extent of modulation by either TPA or TGF-beta over that observed after 24 hr. At both 24 and 72 hr time points TPA appeared to have a greater effect on polypeptide expression in NHBE cells than did TGF-beta. After 24 hr treatment 26 polypeptides were significantly modulated by TPA and TGF-beta. Fifteen polypeptides were specifically affected by TPA, 10 were up-regulated and 5 were down-regulated; 4 polypeptides were modulated by TGF-beta alone (3 up- and 1 down-regulated) while 6 polypeptides were affected by both TPA and TGF-beta. After 72 hr, 38 polypeptides were specifically modulated by TPA, 3 were affected by both TPA and TGF-beta while only one polypeptide was affected only by TGF-beta. Of the 42 polypeptides modulated at 72 hr, 4 (5.85/88 kDa; 6.10/40 kDa; and 5.00/40 kDa; and 5.80/28 kDa) were also observed to exhibit significant quantitative changes at 24 hr.

Publications:

Wirth, P. J., Doniger, J., Thorgeirsson, S. S., DiPaolo, J. A.: Altered polypeptide expression associated with neoplastic transformation in Syrian hamster cells by bisulfite. Cancer Res. 46: 390-399, 1986.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05446-02 LEC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Initiation and Termination of Hepatocyte Proliferation by Serum Factors		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Snorri S. Thorgeirsson	Chief LEC NCI
Others:	Anthony C. Huggett	Visiting Fellow LEC NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Experimental Carcinogenesis		
SECTION Biopolymer Chemistry Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Studies on parabiotic rats and on liver transplantation have suggested that following partial hepatectomy a humoral factor stimulates quiescent hepatocytes to enter the cell cycle. The nature of the serum factors that regulate hepatocyte proliferation has been investigated. A bioassay procedure for the determination of DNA synthesis in primary hepatocyte cultures has been established. This has allowed the purification of a putative hepatocyte growth factor from the serum of hepatectomized rats. In this report we present the following major findings: (1) The partially purified factor isolated from the serum of young Fischer 344 rats produces a dose-dependent stimulation of DNA synthesis in primary hepatocyte cultures. (2) The hepatocyte growth factor appears to be an acid- and heat-labile protein of molecular weight 70-110 KD which has a strong affinity for heparin. (3) The factor also stimulates DNA synthesis in cultures of rat kidney fibroblasts, but is inactive in fetal liver cell cultures and in cultures of hepatoma cell lines. (4) The factor may be a novel growth factor as it has different physical or biological properties compared to known well-characterized growth proteins and polypeptides. (5) There may be a factor(s) present in normal serum which inhibits the activity of the putative hepatocyte growth factor.</p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Snorri S. Thorgeirsson	Chief	LEC NCI
Anthony C. Huggett	Visiting Fellow	LEC NCI

Objectives:

The objective of this project is to isolate and characterize, both biologically and structurally, the serum polypeptides and proteins involved in the stimulation and termination of hepatocyte proliferation following partial hepatectomy. The aim is to investigate the role played by such factors in the neoplastic process.

Methods Employed:

The principal methods employed in these studies include: (1) use of affinity chromatography, ultrafiltration, fast protein liquid chromatography (FPLC), high performance liquid chromatography and two-dimensional polyacrylamide gel electrophoresis to purify polypeptides and proteins from serum obtained from partially hepatectomized Fischer rats; (2) maintenance of primary hepatocyte cultures in defined serum-free medium; (3) fluorimetric assay of cellular DNA and assay of the incorporation of tritiated thymidine into DNA of cells maintained in 96-well microtiter plates; (4) study of the action of these factors on various cell types including normal hepatocytes, preneoplastic and neoplastic hepatocytes and cells from other tissues.

Major Findings:

Previous studies in other laboratories have shown that serum obtained from partially hepatectomized rats is more active in stimulating DNA synthesis in primary cultures than serum obtained from control rats. We are investigating serum from hepatectomized rats in order to determine the components responsible for this difference.

The development of an assay for hepatocyte proliferation has taken place concurrently with the partial purification of a hepatocyte growth factor for rat serum. Hepatocyte plating density was shown to have a marked effect upon the susceptibility of hepatocytes to growth stimulation, with a suppression of DNA synthesis observed at confluent cell density. Culture conditions were modified such that the proliferation assay could be performed using defined media in the absence of serum.

We have achieved partial purification of a factor which stimulates DNA synthesis in primary hepatocyte cultures of rat hepatocytes using heparin-affinity chromatography and gel filtration of serum from normal Fischer rats and from rats 24 hours after partial hepatectomy. A 25-fold increase in DNA synthesis compared to control was observed upon addition of the partially purified serum factor to

the hepatocyte cultures. No difference could be found in the stimulatory activity of heparin-affinity purified fractions obtained from normal and hepatectomized rats indicating that the partially purified serum factor does not solely account for the humoral regenerative stimulus.

The partially purified serum factor has an apparent molecular weight of 70-120 KD. Preliminary characterization has indicated that it is an acid- and heat-labile protein. The factor is active in stimulating DNA synthesis in cultures of both primary hepatocytes and normal rat kidney fibroblasts although it was inactive in hepatoma cell lines and also in a culture of a nonmalignant line of neonatal rat liver epithelial cells. Various well- and partially-characterized growth factors were also examined for their ability to stimulate DNA synthesis in the hepatocyte proliferation assay. Apart from EGF, only endothelial cell growth factor (ECGF) produced a significant stimulation of DNA synthesis. However, it was noted that while EGF had no stimulatory effect upon hepatocytes plated at a high cell density, the partially purified serum factor was able to overcome the cell-cell contact inhibitory effect indicating, to some extent, a different mechanism of growth stimulation. Based on its physical and biological properties the serum hepatocyte growth factor appears to be a novel growth factor.

Publications:

None

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05447-02 LEC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Isolation & Characterization of Proteins from Two-Dimensional Polyacrylamide Gels		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Peter J. Wirth	Expert LEC NCI
Others:	Anthony C. Huggett	Visiting Fellow LEC NCI
	Timothy Benjamin	Chemist LEC NCI
	Preston Grantham	Chemist LEC NCI
	Peter P. Roller	Head, Biopolymer Chemistry Section LEC NCI
	Snorri S. Thorgeirsson	Chief LEC NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Experimental Carcinogenesis		
SECTION Biopolymer Chemistry Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> The purpose of this project is to develop the analytical technology required for the elution and microsequencing of proteins from two-dimensional polyacrylamide gels. Initial work has been focused on the sequence analysis of proteins that are selectively expressed during the course of multistage hepatocellular carcinogenesis. The availability of such a technique will facilitate the construction of DNA probes such that both gene cloning and studies on gene expression can be achieved. Microscale procedures for the isolation of proteins from polyacrylamide gels have been examined. Although electroelution and formic acid extraction from the gel into solution gave a high recovery of protein, sequence analysis and amino acid analysis was greatly complicated by contamination with gel components and N-terminal blocking of the proteins during isolation, such that microquantities of proteins could not be analyzed using these techniques. Electrophoretic techniques were investigated for the transfer of protein to a solid matrix prior to direct gas phase sequencing. As little as 15 pmole of protein has been directly transferred from a gel to derivatized glass fiber paper using this technique. In addition the N-terminal sequence analysis of 29 pmole protein directly applied to derivatized glass fiber paper has been accomplished. Work is currently underway to optimize conditions to allow the direct sequence analysis of microquantities (< 20 pmole) of transblotted protein. In addition methodology for the amino acid sequencing of N-terminally blocked proteins is under development. </p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Peter J. Wirth	Expert	LEC NCI
Anthony C. Huggett	Visiting Fellow	LEC NCI
Timothy Benjamin	Chemist	LEC NCI
Preston H. Grantham	Chemist	LEC NCI
Peter P. Roller	Head, Biopolymer Chemistry Section	LEC NCI
Snorri S. Thorgeirsson	Chief	LEC NCI

Objectives:

The major objective of this project is to isolate and characterize relevant proteins from polyacrylamide gels following preparative two-dimensional gel electrophoresis (2D-PAGE) of relatively crude tissue preparations. The availability of partial protein sequence information will allow the construction of DNA probes which will facilitate both gene cloning and gene expression studies.

Methods Employed:

The principal methods employed are: (1) 2D-PAGE, (2) transblotting of proteins to derivatized glass fiber paper, (3) gas phase amino acid sequence analysis, (4) electroelution of proteins, (5) high performance liquid chromatography, (6) chemical and enzymic digestion of proteins, (7) amino acid analysis, and (8) fast atom bombardment mass spectrometry.

Major Findings:

The computer-assisted two-dimensional gel electrophoresis methodology developed within this laboratory has provided a highly sensitive procedure capable of resolving large numbers of proteins within a sample. This technique has been successfully applied to the investigation of differences in gene expression between normal, preneoplastic and neoplastic tissues. We have focused our studies on the differences in protein patterns obtained from 2D-PAGE analysis of Solt-Farber-induced hepatocellular carcinoma with the patterns obtained from the analysis of normal hepatic tissue. At least four protein spots have been detected in neoplastic tissue which were not observed in normal tissue (mol. wt., PI, location): (A) 57 KD, 6.8, cytosol; (B) 41 KD, 6.25, membrane; (C) 24 Kd, 6.75, membrane; (D) 21 KD, 6.05, membrane.

The isolation and characterization of proteins from 2D-PAGE gels requires the development of a battery of microanalytical techniques due to the small quantity of protein present in a gel spot. Using a sensitive silver stain for polypeptide visualization, spots containing from 0.01 to 1 microgram protein are routinely separated. Our initial investigations have been focused on the development of microscale procedures for gel elution, sample handling, enzymic digestion, peptide purification and amino-terminal amino acid sequencing. Microsequencing technique enabling analysis of amino terminal-blocked proteins are currently under evaluation.

We have demonstrated that electroelution or formic acid extraction produces a high yield of proteins from Coomassie blue-stained polyacrylamide gels. However, amino acid analysis demonstrated considerable sample contamination with amino acids, particularly glycine and serine. Amino acid sequence analysis was successfully performed on 770 pmole albumin extracted from an SDS-polyacrylamide gel. Further studies have indicated that these procedures are not suitable for the isolation and sequence analysis of the small amounts of protein (< 1 microgram) in 2D-gel spots.

The electrophoretic transfer of proteins from polyacrylamide gels to solid matrices prior to sequence analysis was investigated. The use of nitrocellulose membranes was excluded since only very small amounts of sequencable protein could be recovered. Transblotting to derivatized glass fiber paper (GF/C) proved more successful. A blotting efficiency of 86% was achieved for the transfer of 100 pmole albumin from a polyacrylamide gel to quaternary ammonium derivatized GF/C paper, and as little as 15 pmole of albumin has been successfully transblotted. In addition N-terminal amino acid sequence analysis using a gas phase sequenator was successful on 29 pmole of albumin applied to quaternary ammonium glass fiber paper. Concurrently, the optimization of N-terminal amino acid microsequencing employing a gas phase sequenator has been performed.

Methodology for the analysis of N-terminally blocked proteins is currently under development. The enzymic fragmentation of proteins following isolation from polyacrylamide gels prior to HPLC peptide separation and sequencing is being investigated. In addition the purification of proteins from liver samples prior to 2D-PAGE is currently being undertaken.

Publications:

None

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05448-02 LEC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Guanosine Triphosphate Binding Site of ras Protein by NMR and CD Spectroscopy		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: Chien-Hua Niu	Expert	LEC NCI
Others: Kyou-Hoon Han Peter P. Roller	Visiting Fellow Head, Biopolymer Chemistry Section	LEC NCI LEC NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Experimental Carcinogenesis		
SECTION Biopolymer Chemistry Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: <div style="text-align: center;">1.5</div>	PROFESSIONAL: <div style="text-align: center;">1.5</div>	OTHER: <div style="text-align: center;">0</div>
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews </div> <div> <input type="checkbox"/> (b) Human tissues </div> <div> <input checked="" type="checkbox"/> (c) Neither </div> </div>		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> A number of studies indicate that a point mutation at either position 12, 13, 59 or 61 of <u>ras</u> p21 proteins is associated with a fundamental change in their biochemical properties including their ability to transform cells. The main objective of this project is to study the conformational differences between non-transforming and transforming <u>ras</u> p21 proteins as well as the conformational changes upon addition of GTP. Results obtained so far are as follows: (1) Both glycine-containing (Gly-peptide) and valine-containing (Val-peptide) 34 amino acid residue peptides of N-terminal segments of p21 proteins have been synthesized and purified. Their structures were confirmed by mass spectroscopy and peptide sequencing. (2) It is notable that a single amino acid substitution in the N-terminal segment produces a distinct change in the solubility properties. (3) In Tris buffer (pH 7.4), the Gly-peptide adopted a largely beta-sheet structure. However, in 40% trifluoroethanol (TFE), the Gly-peptide showed an increased amount of alpha-helical structure (46%). (4) The Val-peptide in ammonium acetate buffer (pH 7.4) adopted a greater amount of alpha-helical structure relative to that of the Gly-peptide in Tris buffer. (5) The addition of GTP to the Gly-peptide induces a larger amount of change in its conformation. In contrast, upon addition of nucleotides to the Val-peptide solution, little overall conformational change was noted. (6) When the Gly-peptide was added to the solution containing GTP and SDS, the line widths of all three P-31 NMR signals, alpha, beta, and gamma, were broadened (10 Hz for beta and gamma, and 5 Hz for alpha). The result implies that there is a complex formation between GTP and the Gly-peptide. (7) Equilibrium dialysis experiments indicate that the binding strength of the Gly-peptide and Val-peptide with GTP are comparable to those of intact p21 proteins. The model peptides bind GTP and ATP indiscriminately. (8) N-Terminal segments of p21 proteins are involved in the hydrolysis of GTP. </p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Chien-Hua Niu	Expert	LEC NCI
Kyou-Hoon Han	Visiting Fellow	LEC NCI
Peter P. Roller	Head, Biopolymer Chemistry Section	LEC NCI

Objectives:

The main objectives of the project are (1) to study the conformational differences between nontransforming and transforming ras p21 proteins, (2) to investigate the conformational changes in p21 proteins upon binding to nucleotides by various spectroscopic methods, (3) to locate the GTP binding site in p21 proteins, and (4) to identify the amino acid residues involved in the hydrolysis of GTP in p21 proteins. Our overall goal is to understand how the amino acid substitutions at position 12 in ras p21 proteins produce such profound and powerful changes in their transformation properties.

Methods Employed:

The principal methods employed are (1) peptide synthesis and purification, (2) nuclear magnetic resonance (NMR), (3) circular dichroism (CD), (4) equilibrium dialysis, and (5) calculations of binding constants.

Major Findings:

(1) Both glycine-containing (Gly-peptide) and valine-containing (Val-peptide) 34 amino acid residues of N-terminal segments of ras p21 proteins have been purified by reverse phase liquid chromatography (RP-HPLC). Their structure was confirmed by FAB mass spectroscopy and peptide sequencing.

(2) It is notable that a single amino acid substitution in the N-terminal segment of p21 proteins produces a great change in the solubility properties. The Gly-peptide was soluble in acidic solvents, Tris buffer, and 40% trifluoroethanol (TFE), but not in ammonium acetate buffer. In contrast, among these solvents, the Val-peptide could dissolve only in ammonium acetate buffer.

(3) In Tris buffer (pH 7.4), the Gly-peptide adopted a largely beta-sheet conformation (51%) with 49% random coil. However, in 40% TFE solution, the Gly-peptide showed an increased amount of alpha-helical structure (46%). This result indicates that the conformation of the Gly-peptide depends upon the environment in which it is dissolved.

(4) The Val-peptide in ammonium acetate buffer (pH 7.4) adopted a greater amount of alpha-helical structure relative to that of the Gly-peptide in Tris buffer. The result indicates that substitution of glycine with valine in the N-terminal segment of p21 proteins change the structural conformation.

(5) The addition of GTP to the Gly-peptide induces a larger amount of change in its conformation. In TFE solution, the peptide conformation appeared to change

from alpha-helix to the beta-sheet structure. In Tris buffer, the conformation of the peptide shifted to the random coil structure from beta-sheet structure. In contrast, upon addition of nucleotides to the Val-peptide solution, little overall conformational change was substantially altered. These findings imply that substitution of glycine with valine in p21 proteins causes a restriction of local conformation near position 12 which may produce a transforming capability.

(6) Upon addition of the Gly-peptide to either GTP or ATP solution containing SDS, the line widths of all three phosphorous-31 signals, alpha, beta and gamma, were broadened (10 Hz for beta and gamma, and 5 Hz for alpha). The result implies that there is a complex formation between nucleotides and the Gly-peptide.

(7) The results obtained from equilibrium dialysis experiments indicate that both GTP and ATP bind to the Gly-peptide with binding constants of 7.1×10^6 and $5.3 \times 10^6 \text{ M}^{-1}$, respectively, and to the Val-peptide with constants of 6.5×10^6 and $1.6 \times 10^6 \text{ M}^{-1}$, respectively. These studies reveal that the model peptides containing 34 amino acid residues of the N-terminal segment of the p21 protein bind GTP and ATP indiscriminately.

(8) When GTP was added to the Gly-peptide solution containing magnesium ions, the P-31 NMR signal intensities of inorganic phosphate and GDP were gradually increased, while that of the GTP signal slowly decreased. The result indicates that the N-terminal segment of p21 proteins may be involved in the hydrolysis of GTP.

Publications:

None

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05449-02 LEC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Conformational Studies on Epidermal Growth Factor and Transforming Growth Factor		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: Chien-Hua Niu Others: Kyou-Hoon Han Peter P. Roller Snorri S. Thorgeirsson	Expert Visiting Fellow Head, Biopolymer Chemistry Section Chief	LEC NCI LEC NCI LEC NCI LEC NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Experimental Carcinogenesis		
SECTION Biopolymer Chemistry Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Epidermal growth factor (EGF-beta) and transforming growth factor (TGF-alpha) are structurally related and exhibit similar activities in competition for binding to the EGF receptor, stimulation of DNA synthesis, and cell proliferation. The objective of the project is to study the mechanism of their mitogenic activities based on their molecular conformations. The conformation of naturally occurring and synthetic analogues of EGF and TGF-alpha will be studied by nuclear magnetic resonance (NMR), circular dichroism (CD), laser Raman, and infrared spectroscopic methods. The radioreceptor assay and the mitogenic assay will be used for testing biological activities of the synthetic analogues. At present, the research is focused on the following areas: (1) cyclization of synthetic peptides through disulfide linkage by high dilution method, (2) purification of cyclic peptides by gel chromatography and reversed phase HPLC systems, (3) examination of the biological activities of these synthetic peptides, (4) conformational studies of synthetic analogues of EGF and TGF-alpha by modern two-dimensional NMR techniques. Results obtained so far include: (1) Isolation and purification of human EGF from urine has been achieved by ethanol precipitation, ion-exchange chromatography, gel filtration, and FPLC. Fractions active in stimulating DNA synthesis in primary rat hepatocytes were obtained. The radioreceptor assay also was used to confirm biological activities of the active fraction. However, a minute quantity of human EGF was isolated from urine. (2) Two cyclic peptides, analogues of human TGF-alpha, c(C-R-F-L-V-Q-E-D-K-P-A-C) and c(C-F-H-G-T-A-R-F-L-V-Q-E-D-K-P-A-C), have been successfully synthesized by high dilution method. The structures of cyclic peptides were confirmed by FAB mass spectrometry. (3) Conformational analysis of 17-mer is now underway.		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Chien-Hua Niu	Expert	LEC NCI
Kyou-Hoon Han	Visiting Fellow	LEC NCI
Peter P. Roller	Head, Biopolymer Chemistry Section	LEC NCI
Snorri S. Thorgeirsson	Chief	LEC NCI

Objectives:

The main objectives of this project are: (1) to study the molecular conformation of naturally occurring epidermal growth factor (EGF-beta) and transforming growth factor (TGF-alpha) as well as their synthetic analogues using modern nuclear magnetic resonance (NMR), laser Raman, and x-ray diffraction techniques; (2) to locate the binding site of the EGF receptor; (3) to design and develop antagonists against TGF-alpha for therapeutic use; and (4) to study EGF-receptor complex interaction by carbon-13 NMR spectroscopy with isotopically enriched preparations.

Methods Employed:

The principal methods employed are (1) peptide synthesis; (2) binding assays; (3) nuclear magnetic resonance (NMR), circular dichroism, infrared, and laser Raman spectroscopy; (4) computer graphic techniques; (5) crystallization of peptides, and (6) x-ray diffraction.

Major Findings:

(1) Attempts were made to isolate human EGF for the physicochemical studies from commercially available crude material. A minute quantity of human EGF was isolated and purified from human urine by ethanol precipitation; ion-exchange chromatography, gel filtration, and FPLC. The resulting EGF containing fractions were determined with bioassays of stimulating DNA synthesis in primary rat hepatocytes and also with the radioreceptor assay on the A431 carcinoma cell line. It became clear that this approach will not have provided sufficient material for our intended studies; however, the material will serve as a positive control for testing synthetic variants of EGF.

(2) Two cyclic peptides, c(C-R-F-L-V-Q-E-D-K-P-A-C) and c(C-F-H-G-T-A-R-F-L-V-Q-E-D-K-P-A-K), both analogs of human TGF-alpha, have been successfully synthesized and purified. Intramolecular cyclization was achieved by linking the sulfhydryl groups of 2 cysteines with the oxidizing agent, potassium ferricyanide, in a very dilute solution at pH 7.4. The structures of cyclic peptides were confirmed by FAB mass spectrometry. It is known that in the natural growth factors these peptide segments form the central cyclic disulfide linked portion, and possibly the receptor binding sites. These peptides will be used for our physicochemical and biological studies.

(3) Conformational studies of these cyclic peptides using NMR and laser Raman spectroscopy are now underway.

Publications:

None

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05450-02 LEC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Chromatin Structure and Steroid Hormone Action		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Gordon L. Hager	Head, Hormone Action & Oncogenesis Section LEC NCI
Others:	Helene Richard-Foy	Guest Researcher LEC NCI
	Diana S. Berard	Microbiologist LEC NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Experimental Carcinogenesis		
SECTION Hormone Action and Oncogenesis Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.9	1.5	0.4
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> The mouse mammary tumor virus long terminal repeat (MMTV LTR) contains a glucocorticoid responsive transcriptional regulatory element. Using a 69% transforming fragment of bovine papilloma virus (BPV) Type I, we have amplified the MMTV LTR in cultured murine cells. The LTR-BPV molecules present in these cells occur exclusively as unintegrated, extrachromosomal episomes, with a copy number up to 300/cell. Minichromosomes containing the MMTV LTR are capable of selectively binding the glucocorticoid receptor, whereas minichromosomes containing only BPV do not. Specific regions of the minichromosomes fractionate differentially when analyzed after micrococcal nuclease digestion for distribution in fractions classically defined as "active" and "inactive" chromatin. LTR sequences (containing transcriptional control regions) fractionate as the bulk DNA, while sequences transcribed from the LTR, at least in the presence of hormone, are almost exclusively present in the "active" fraction. The nucleosome positioning within the LTR has been investigated by the indirect end labeling technique, utilizing micrococcal nuclease as a probe for inter-nucleosomal sequences. The pattern of digestion with chromatin is clearly different from that observed with DNA. A repeating series of cuts is obtained with chromatin, with a periodicity of approximately 190 bp. We conclude that nucleosomes are phased in the MMTV LTR from the 5' end to position -250 from the CAP site. The phasing is unchanged whether or not the hormone is present. A relatively broad region of the LTR (-100 to -180) becomes hypersensitive to DNase I upon stimulation of transcription with dexamethasone. This region corresponds to the potential localization of a nucleosome and is located between the regions protected by the hormone-receptor complex in footprinting studies. It is therefore possible that "remodelling" of nucleosome structure is integral to the mechanism of stimulation of transcription by glucocorticoid hormones. </p>		

PROJECT DESCRIPTIONName, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Gordon L. Hager	Head, Hormone Action & Oncogenesis Section	LEC NCI
Helene Richard-Foy	Guest Researcher	LEC NCI
Diana S. Berard	Microbiologist	LEC NCI

Objectives:

Chimeras between bovine papilloma virus (BPV) and the mouse mammary tumor virus LTR are to be constructed. Cell lines are to be characterized that carry uniquely episomal chimeric DNA and in which hormone regulation at the episomal promoter can be demonstrated.

Episomal chimeric DNA will be isolated from these cells as intact nucleoprotein particles. These particles will be used in nuclease digestion studies to characterize chromatin organization in the vicinity of the hormone-regulated promoter and to identify changes in structure associated with hormone action. Binding localizations of regulatory proteins are to be determined by footprint analysis.

Mutants in the hormone response developed and characterized by oligo-scanning mutagenesis will be transferred to the episomal vectors, and the mutational effects on chromatin structure characterized.

Methods Employed:

Minichromosomes to be used for nuclease digestion and in vitro transcription templates will be purified by low salt procedures that are unlikely to damage chromatin structure. These procedures will depend on the small size of the episomes, but they must also be able to dissociate the higher orders of structure present in the nuclear matrix including so-called nuclear scaffolding. Chelators of specific ions may be useful in this process.

Minichromosomes used for run-off transcription are prepared from nuclei of cells containing LTR episomes by ammonium sulfate extraction. This procedure relies on the small size of these episomes as compared to chromosomal DNA. These particles are then incubated with radioactive with ribonucleotides. The RNA produced is analyzed by hybridization to single-strand probes representing the sequences present in the chimeric BPV LTR episomes.

Isolation of steady-state levels of RNA and S1 mapping of this RNA using end-labeled probes are accomplished using published procedures.

Major Findings:

The central thrust of the work described here was to establish a genetically non-complex system for the study of transcriptional regulation by glucocorticoid hormones and to initiate an examination of the role of epigenetic organization in this regulatory process. We have established that the viral LTR encodes the complete primary genetic structure for hormone responsiveness. The regulatory

effects we have described occur in cell lines whose extrachromosomal BPV-specific molecular populations are homogenous and contain only those sequences deliberately introduced into the constructions for the purpose of selection and replication. For the first time we have demonstrated a regulation of transcription occurring in a well-defined environment at the extrachromosomal promoters, excluding the participation of uncharacterized genetic elements. This model allows us to study the chromatin structure of a hormonally regulated element and to demonstrate a difference in nucleosome organization of the regulatory region compared to transcribed sequences. Upon hormonal stimulation the chromatin structure of the region upstream of the TATA box undergoes changes, resulting in the appearance of a DNase I hypersensitive site. These results open the way to studies leading to understanding the role of epigenetic organization in the mechanism of steroid hormone action.

Publications:

Green, L., Schlaffer, I., Wright, K., Moreno, M. L., Berard, D., Stein, J., Hager, G. L., and Stein, G.: Cell cycle-dependent expression of a stable episomal human histone gene in a mouse cell. Proc. Natl. Acad. Sci. USA 83: 2315-2319, 1986.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05451-02 LEC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Hepatocellular Carcinoma: Expression of Retroviral Associated Oncogenes		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Brian E. Huber	Senior Staff Fellow LEC NCI
Others:	Snorri S. Thorgeirsson	Chief LEC NCI
	Carole A. Heilman	Senior Staff Fellow LEC NCI
	Irene B. Glowinski	Senior Staff Fellow LEC NCI
	Susan H. Sorrell	Chemist LEC NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Experimental Carcinogenesis		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	1.2	1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> The objective of this project is to characterize the role of retroviral associated oncogenes in both the formation and/or maintenance of hepatocellular carcinoma (HCC). The experimental systems presently under study are (a) a human hepatoma cell line, HEP G2 and (b) an in vivo rat model for chemically-induced hepatocellular carcinoma. The results obtained with the HEP G2 study include the tumorigenic characterization of this cell line which may be associated with elevated levels of <u>c-myc</u> transcripts coupled to the expression of an "activated" N-ras gene. The greatly increased steady-state level of <u>c-myc</u> transcripts in HEP G2 cells is the result of increased gene transcription and not the result of a specific stabilization of the c-myc message. Early preneoplastic changes were examined by isolating preneoplastic foci hepatocytes (which lack the cell-surface asialoglycoprotein receptor) in addition to later stage preneoplastic nodules and hepatocellular carcinomas. Utilizing this system, we have examined the expression of genes that are associated with hepatocyte proliferation [ornithine decarboxylase (ODC)], differentiation [albumin (ALB)] and oncofetal development [<u>myc</u>, <u>raf</u>, <u>Ha-ras</u>, P53 and alpha-fetoprotein (AFP)] during the development of HCC in carcinogen-treated Fischer rats by Northern blot analysis. ODC transcripts were not increased in liver foci but were increased in nodules and HCC. <u>Myc</u> transcripts, however, were increased in liver foci, nodules and HCC. AFP and P53 transcripts were not detected in liver foci, barely detectable in nodules and variably expressed in HCC, whereas ALB transcripts were decreased by approximately 50% in all HCC. <u>Ha-ras</u> and <u>raf</u> transcripts were variably expressed in all samples and could be correlated with a particular stage of HCC development. The data suggest that increases in steady-state levels of <u>myc</u> transcripts may be an early event in HCC development and may not be related to hepatocyte proliferation, but rather to alterations in the differentiation state. </p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Brian E. Huber	Senior Staff Fellow	LEC NCI
Snorri S. Thorgeirsson	Chief	LEC NCI
Carole A. Heilman	Senior Staff Fellow	LEC NCI
Irene B. Glowinski	Senior Staff Fellow	LEC NCI
Susan Sorrell	Chemist	LEC NCI

Objectives:

The major objective of this project is to characterize the role of retroviral associated oncogenes in both the formation and/or maintenance of hepatocellular carcinoma. To realize this objective two experimental systems are being utilized. The first system employs a human hepatoma cell line, HEP G2, which provides a model where oncogene expression can be examined in the transformed human hepatocyte. Utilizing this cell system, topics such as the mechanism(s) of c-oncogene activation, the interaction between c-oncogenes, and the role of c-oncogene in the transformation process are being investigated. The second system under study is an in vivo rat model for chemically induced hepatocellular carcinoma where gene expression can be investigated during the transition from normal hepatocytes to preneoplastic foci, preneoplastic nodules and neoplastic tumors.

Methods Employed:

- Methods used in these studies include: tissue culture techniques; liver perfusion radioimmunoassay; differential centrifugation and chromatographic techniques; radioisotope measurements using tritium, carbon-14, phosphorus-32 and sulfur-35; enzyme assays involving radiometric and spectrophotometric determinations; computer-assisted two-dimensional gel electrophoresis; and molecular biological techniques including Northern, Southern and Western blotting.

Major Findings:

A. Oncogene expression in a human hepatoma cell line, HEP G2. HEP G2 is a human cell line derived from a primary hepatocellular carcinoma. We have previously demonstrated that this cell line produces nonencapsulated, highly invasive adenocarcinomas when injected subcutaneously into athymic nude mice. This HEP G2 system, using normal human liver as a control, provides a model for identifying the genes and gene products that are associated with the tumorigenic phenotype. As a first step, expression of proto-oncogenes (cellular oncogenes) implicated in other human malignancies is being examined. We have previously shown both the transcription and translational presence of a mutated N-ras gene in HEP G2 cells (Huber et al., Cancer Res. 45: 4322 and Notario et al., Cancer Cells 425, Cold Spring Harbor Laboratory, 1984).

Coupled to expression of an activated N-ras oncogene, we have reported that the steady-state level of c-myc transcripts are greatly elevated in HEP G2 cells compared to normal human liver.

The greatly increased steady-state level of c-myc transcripts in HEP G2 cells is the result of increased gene transcription and not the result of a specific stabilization of the c-myc message, since the half-life of this message in HEP G2 cells is 15 to 30 min. A 15 min pretreatment with cycloheximide increased the half-life of the c-myc transcript by at least fivefold, which suggests the extreme lability that the HEP G2 c-myc message is the result of a short-lived protein molecule. The increased c-myc expression is identical in log phase and non-dividing confluent cultures. Constitutive expression of the c-myc gene is not the result of c-myc gene amplification, hepatitis B virus integration into the HEP G2 genome, or gross chromosomal aberrations of chromosome 8.

We are presently examining qualitative changes in transcriptional promoters in the HEP G2 c-myc gene by S1 nuclease mapping and c-myc rearrangements by Southern blot analysis. In addition, the functional significance of the HEP G2 c-myc gene in the transformation process is being examined by interrupting endogenous c-myc function with an anti-sense c-myc transcript.

B. Gene expression in the progression of chemically induced hepatocellular carcinoma in the rat. A characteristic feature in the development of HCC is the appearance of microscopic foci of altered hepatocytes which can progress to pre-neoplastic nodules, some of which may undergo additional alterations into primary hepatomas. However, the sequence of cellular events essential at each stage in the progressive development of HCC are not yet understood. We have examined the expression of genes that are associated with hepatocyte proliferation [ornithine decarboxylase (ODC)], differentiation [albumin (ALB)], and oncofetal development [myc, raf, Ha-ras, P53 and alpha-fetoprotein (AFP)] during the development of HCC in carcinogen-treated Fischer rats (Solt and Farber, Nature 263: 701, 1976) by Northern blot analysis using poly(A) RNA from preneoplastic foci and nodules as well as primary HCCs. Cells composing preneoplastic foci were isolated by their inability to bind to tissue culture plates coated with asialofetuin, since these focal cell populations lack the cell surface asialoglycoprotein receptor (Evarts et al., Cancer Res. 44: 5718, 1984). ODC transcripts were not increased in liver foci but were increased in nodules and HCC. Myc transcripts, however, were increased in liver foci, nodules and HCC. AFP and P53 transcripts were not detected in liver foci, barely detectable in nodules and variably expressed in HCC, whereas ALB transcripts were decreased by approximately 50% in all HCC.

Ha-ras and raf transcripts were variably expressed in all samples and could not be correlated with a particular stage of HCC development. The data suggest that increases in steady-state levels of myc transcripts may be an early event in HCC development and may not be related to hepatocyte proliferation, but rather to alterations in the differentiation state.

In addition, DNA transfection experiments were performed to identify and isolate a dominant-acting transforming gene(s) in chemically induced hepatocellular carcinoma. DNA obtained from a primary hepatocellular was not able to transform NIH 3T3 cells but did produce transformed foci when transfected into EL2 cells (rat fibroblasts). These primary EL2 cell transfectants were able to grow in soft agar. We are presently attempting to identify this dominant-acting transforming gene.

Publications:

Huber, B. E., Dearfield, K. L., Williams, J. R., Heilman, C. A. and Thorgeirsson, S. S.: The tumorigenicity and transcriptional modulation of c-myc and N-ras oncogenes in a human hepatoma cell line. Cancer Res. 45: 4322-4329, 1985.

Huber, B. E., Glowinski, I. and Thorgeirsson, S. S.: Transcriptional and post-transcriptional regulation of the asialoglycoprotein receptor in normal and neoplastic liver. J. Biol. Chem. (In Press).

Huber, B. E., Heilman, C. A., Wirth, P. J., Miller, M. and Thorgeirson, S. S.: Studies in gene transcription and translation in regenerating rat liver: Cloning of regenerative specific sequences. Hepatology 6: 209-219, 1986.

McManus, M. E., Minchin, R. F., Schwartz, D. M., Wirth, P. J. and Huber, B. E.: Induction by phenobarbital in McA-RH7777 rat hepatoma cells of a polycyclic hydrocarbon inducible cytochrome P450. Biochem. Biophys. Res. Commun. 137: 120-127, 1986.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05452-02 LEC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Gene Expression and Development in Transgenic Mice		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Su-yun Chung	Senior Staff Fellow LEC NCI
Others:	Snorri S. Thorgeirsson	Chief LEC NCI
	Miriam Falzon	Visiting Fellow LEC NCI
	Shu-hua Yu	Visiting Fellow LEC NCI
	Nancy Sanderson	Chemist LEC NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Experimental Carcinogenesis		
SECTION Cell Biology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.1	3.1	1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The overall objective of this project is to exploit the transgenic mouse system by introducing natural or manipulated gene sequences into the germ line of an animal and to alter its phenotype and genetic background. This system provides a new way of investigating tissue-specific and developmental stage-specific regulation of gene expression. The current research is focused on two classes of genes that may be associated, initially, with the multistage process of murine liver tumorigenesis: (1) the known oncogenes, <i>myc</i>, <i>ras</i> and SV40 T antigen, and (2) the developmental expression of a family of genes that contain sequences homologous to the <i>Drosophila</i> homeobox. Work in the past year has focused on setting up the transgenic mouse system and on cloning putative murine homeotic genes. We have succeeded in making transgenic mice following micro-injection of SV40 T antigen under the control of the metallothionin promoter. The genomic localization and expression of the introduced sequences are being characterized. Other oncogene constructs are currently being employed to obtain new transgenic mice lines. We have also obtained six independent, putative homeotic clones from a rat genomic library. DNA sequence analysis indicates these clones contain sequences exhibiting greater than 90% homology to the consensus homeobox sequence. We have observed expression of these genes in spinal cord, brain and embryo.</p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Su-yun Chung	Senior Staff Fellow	LEC NCI
Snorri S. Thorgeirsson	Chief	LEC NCI
Miriam Falzon	Visiting Fellow	LEC NCI
Shu-hua Yu	Visiting Fellow	LEC NCI
Nancy Sanderson	Chemist	LEC NCI

Objectives:

New developments in the past few years have made it possible to generate transgenic mice which carry introduced foreign genes in their germ line genome. The technique involves microinjecting cloned DNA fragments into the pronucleus of a fertilized egg. A number of laboratories have reported that the introduced genes integrated into the host genome are stably transmitted to future generations following Mendelian inheritance. Many of the introduced genes are expressed in a tissue-specific manner and the patterns of gene expression are transmitted to the offspring in some lines of transgenic animals. The overall objectives of this study are to exploit the transgenic mouse system to introduce natural or manipulated gene sequences into the germ line of an animal and to alter its phenotype and genetic background. This provides us a new way to investigate tissue-specific and developmental stage-specific regulation of gene expression. Furthermore, the interaction of the introduced gene and its biological effects on the host animal can be monitored throughout normal and malignant development from embryogenesis to adulthood. The current proposal focuses on two classes of genes that may be intimately associated with the multistage process of tumorigenesis in liver. Experiments on these classes of genes are described separately.

(1) We will introduce the known oncogenes, myc, ras and SV40 T antigen, under the control of a liver-specific promoter system into the transgenic mice. The potential role in a multistep process and its effect on liver tumor development of each oncogene will be investigated.

(2) We will monitor the developmental expression of a family of genes that contain sequences homologous to the Drosophila homeobox in normal and transgenic mice. The ultimate goal is to look for an interplay among genes regulating development and genes affecting oncogenesis.

Methods Employed:

(1) Recombinant plasmid constructions and other recombinant DNA techniques, (2) DNA sequencing analysis, (3) microinjection, (4) gel electrophoresis and nucleic acid hybridization analysis, and (5) histology and in situ hybridization.

Major Findings:

(1) We have created transgenic mice lines following microinjection of the SV40 T antigen under the control of the metallothionin promoter. The genomic localization and expression of the introduced sequences are being characterized.

- (2) We have cloned six independent, putative rat homeotic genes.
- (3) DNA sequencing analysis indicates that the rat homeobox is more than 90% homologous to the consensus Drosophila and mouse homeobox.
- (4) The sequence flanking the homeobox is highly divergent.
- (5) The homeobox-containing region is expressed at a high level in brain, spinal cord and embryos.

Publications:

None

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05453-02 LEC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Genetic Determinants in Chemical Hepatocarcinogenesis		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Snorri S. Thorgeirsson	Chief LEC NCI
Others:	Ryuichi Konno Ritva P. Evarts Su-yun Chung Gordon L. Hager	Visiting Fellow Veterinary Medical Officer Senior Staff Fellow Head, Hormone Action & Oncogenesis Section LEC NCI LEC NCI LEC NCI LEC NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Experimental Carcinogenesis		
SECTION Chemical Carcinogenesis Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.3	0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The main objective of this project is to define the genetic determinants for the initiation stage in hepatocarcinogenesis and subsequent evolution of liver tumors that are brought about by chemical carcinogens and other cancer causing agents. The principal lesions that develop in the rat liver as a result of initiation-promotion protocols are foci of altered hepatocytes. Initiation of these foci by a variety of hepatocarcinogens has been shown to follow an apparent first order dose response, suggesting that the foci are a clonal expansion of the initiated cell. Consequently the phenotype of initiation should be completely represented by the foci of altered hepatocytes. To test this hypothesis we have started a series of experiments in which activated retroviral-associated oncogenes are transfected into a nontumorigenic rat liver epithelial cell line and primary rat hepatocytes, and the phenotypic changes determined. The research is currently focused on: (1) transfecting rat liver cells with retroviral associated oncogenes, (2) construction of a retroviral vector-based transfection system to introduce selected oncogenes into primary hepatocytes, and (3) construction of cDNA libraries from normal and neoplastic rat liver.</p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Snorri S. Thorgeirsson	Chief	LEC NCI
Ryuichi Konno	Visiting Fellow	LEC NCI
Ritva P. Evarts	Veterinary Medical Officer	LEC NCI
Sy-yun Chung	Senior Stanf Fellow	LEC NCI
Gordon L. Hager	Head, Hormone Action & Oncogenesis Section	LEC NCI

Objectives:

The main objective of this project is to define the genetic determinants for the initiation stage in hepatocarcinogenesis and subsequent evolution of liver tumors that are brought about by chemical carcinogens and other cancer causing agents. We then plan to examine the in vivo progression of these initiated cells when subjected to a variety of promotional stimuli (phenobarbital, TCDD, etc.). We will employ the rat liver and established normal rat hepatocyte cell line as a model for these studies.

The research is currently focused on: (1) transfecting normal rat hepatocyte (established cell line) with molecular chimeras of MMTV-v-ras and other retroviral-associated oncogenes that can be driven by glucocorticoid hormones and is known to transform 3T3 mouse fibroblasts; (2) determine if morphologic transformation of the transfected hepatocytes is realized upon activation of the v-ras gene by dexamethasone; (3) characterize the cytomorphological and cytochemical changes, should transformation occur, and contrast these changes with those observed with chemically initiated hepatocytes; (4) examine the response of these virally transformed cells to known promoters of chemically induced hepatocarcinogenesis in both anchorage-dependent and independent conditions, growth inhibitors and growth factors known to act upon rat hepatocytes, and (5) transplant the virally transformed hepatocytes into isogenic hosts (liver and anterior chamber of the eye) and characterize the growth and progression toward primary tumor formation under stimuli of glucocorticoid and/or liver tumor promoters.

Methods Employed:

Method used in these studies include: tissue culture techniques, radioisotopic measurements, enzyme assays, histochemical and immunohistochemical methods, and recombinant and molecular technology including DNA and RNA preparation, Northern and Southern blotting, construction of cDNA, genome libraries and nucleic acid hybridization, and construction of retroviral vector systems.

Major Findings:

1. Characterization of normal rat epithelial cells (FNRLM). We have employed a rat liver epithelial cell line (FNRLM) for our initial transfection experiments. This liver epithelial cell line was established from a ten-day old male Fischer (F344) rat by the methods of Herrings et al. (In Vitro 19: 576, 1983) and maintained as monolayer cultures. This cell line has a diploid karyotype and does not express an increased level of GGT; it only demonstrates anchorage-independent

growth in the presence of high (> 10 ng/ml) concentrations of EGF. Furthermore, the FNRLM cells are not tumorigenic when transplanted into an isogenic host. The FNRLM cells, as well as primary hepatocytes, are very sensitive to the growth inhibitory effect of TGF-beta, whereas the aflatoxin B₁-transformed cell line derived from FNRLM cells is resistant to this growth inhibition of TGF-beta. We have also examined the expression of albumin, alpha-fetoprotein, Ha-ras and myc genes in the FNRLM cells. No, or very little, expression of albumin was observed, whereas alpha-fetoprotein was expressed at high level. The Ha-ras and myc genes were expressed at very low levels in the FNRLM cells.

2. Transfection of the FNRLM cells with MMTV-v-ras and MMTV-H3myc. The MMTV-myc construct (kindly provided by Dr. Philip Leder, Harvard Medical School, Boston, MA) contains the MMTV LTR and most of the c-myc gene with the exception of the normal 3' end of the gene (Cell 38: 627,1984). These fusion genes were cotransfected into FNRLM cells with a plasmid containing a neomycin-resistance gene by the calcium phosphate precipitation method. After the transfection, neomycin-resistant transfectants were selected. Several clones have been isolated and are currently being characterized.

Publications:

None

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05454-02 LEC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Chemical Transformation of Human Lymphoblastoid Cell Lines		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Snorri S. Thorgeirsson	Chief LEC NCI
Others:	Dana Kessler	Microbiologist LEC NCI
	Carole A. Heilman	Senior Staff Fellow LEC NCI
	Jeff Cossman	Sr. Assistant Surgeon CP NCI
	George E. Mark	Expert LHC NCI
	Maria G. Tsokos	Expert LP NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Experimental Carcinogenesis		
SECTION Chemical Carcinogenesis Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	0.3	1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> The object of this project is to characterize the nature of chemically induced transition from a benign hyperproliferative to a malignant state in Epstein-Barr virus (EBV) immortalized human lymphocytes. Treatment with N-acetoxy-2-acetylaminofluorene (N-OAc-AAF), a potent frameshift mutagen, induced conversion of the EBV immortalized lymphocytes into high grade "immunoblastic lymphomas" upon injection into athymic mice, whereas injection of the untreated, original cells did not. The tumor cells were all of the B-cell lineage. High molecular weight DNA from the chemically transformed EBV immortalized human cord blood lymphocytes was co-transfected with pSV2Neo into NIH 3T3 cells. After 2 weeks selection in 250 micrograms/ml G418, 10 foci were cloned out and expanded. These foci were positive for soft agar growth and gave rise to fibrosarcomas within 2 weeks in athymic mice. The control NIH 3T3 cells were incapable of anchorage-independent growth or tumor formation. Subsequent Southern analysis revealed that the primary transfected 3T3 cells contained approximately 95 kbp of human DNA as indicated by hybridization to the human repeat sequence, Blue 8. Screening of the foci for various oncogenes revealed that the 3' end of c-raf-1 was present in the transfected cells. This constitutes the first report of chemical activation of the c-raf-1 oncogene in human cell lines. </p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Snorri S. Thorgeirsson	Chief	LEC NCI
Dana Kessler	Microbiologist	LEC NCI
Carole A. Heilman	Senior Staff Fellow	LEC NCI
Jeff Cossman	Sr. Assistant Surgeon	CP NCI
George E. Mark	Expert	LHC NCI
Maria G. Tsokos	Expert	LP NCI

Objectives:

A considerable body of data from both animal experiments and epidemiologic studies provide strong evidence that cancer induction requires a minimum of two stages or events for the unidirectional transition from a normal to a transformed state. A model for naturally occurring human cancer has been proposed in which the first event in the neoplastic process leads to an improperly controlled proliferation of cells (i.e., initiated cells), while the second event results in the commitment of the cells to develop into a clinically apparent cancer (JNCI 66: 1037-1052, 1981).

The objective of this project is to examine the nature of the second event (i.e., the transition from initiation to malignant transformation) in the context of chemically induced neoplasia. We have employed the Epstein-Barr virus (EBV), immortalized human cord blood lymphocytes (CB) and a human peripheral blood lymphocyte culture derived from an EBV-positive patient with infectious mononucleosis (Eckert) as the cell population that has sustained the "first" event in the neoplastic process. These cells have acquired the capacity to grow indefinitely in vitro, and may, therefore, represent the "initiated" cell. Since the natural history, including cytogenetic changes as well as oncogene activation, of the EBV-carrying African form of human Burkitt's lymphoma has been extensively documented, it provides an excellent source of comparison as to the possible etiological role of chemical carcinogens in this disease as well as to the nature of the transition from initiated to transformed cells.

Methods Employed:

Methods used in these studies include: tissue culture techniques; differentiated centrifugation and chromatographic techniques; radioisotopic measurements using tritium, carbon-14, phosphorus-32 and iodine-125; cell surface analysis by fluorescence-activated cell sorting; chromosomal analysis; and recombinant and molecular technology including DNA and RNA preparations, Northern and Southern blotting, and nucleic acid hybridization.

Major Findings:

High molecular weight DNA from in vitro N-Ac-AAF chemically transformed EBV immortalized human cord blood lymphocytes was co-transfected with pSV₂Neo into NIH 3T3 cells. After 2 weeks selection in 250 micrograms/ml G418, 10 foci were cloned out and expanded. These foci were positive for soft agar growth and

10⁶ cells injected subcutaneously into athymic mice gave rise to fibrosarcomas within 2 weeks. The control NIH 3T3 cells were incapable of anchorage-independent growth or tumor formation. Subsequent Southern analysis revealed that the primary transfected 3T3 cells contained approximately 95 kbp of human DNA as indicated by hybridization to the human repeat sequence, Blue 8. Screening of the foci for various oncogenes revealed that the 3' end of c-raf-1 was present in the transfected cells. Indirect immunofluorescence reveals the cytoplasmic presence of the c-raf-1 protein in the transfected cells. Presently further mapping of the oncogene in the transfected cells is being conducted, as is Northern analysis for c-raf-1 in the transfected cells. A secondary round of transfection has been initiated. This constitutes the first report of chemical activation of the c-raf-1 oncogene in human cell lines.

Publications:

Kessler, D., Heilman, C. A., Cossman, J., Maguire, R. T. and Thorgeirsson, S. S.: Transformation of EBV immortalized human B cells by chemical carcinogens. Cancer Res. (In Press).

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05495-01 LEC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Amino Acid at the Suppression Site in Rabbit Beta-Globin Readthrough Protein		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Dolph L. Hatfield	Research Biologist LEC NCI
Others:	Snorri S. Thorgeirsson	Chief LEC NCI
	Michael Bustin	Research Chemist LMC NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Experimental Carcinogenesis		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Rabbit beta-globin readthrough protein is the only naturally occurring read-through protein in higher eukaryotes which does not involve a viral system. Since suppressor tRNAs have been used in gene therapy experiments and have been implicated in inhibiting viral expression, the readthrough protein has been isolated from rabbit reticulocytes in order to identify the amino acid at the suppression site and, therefore, to characterize the nonsense suppressor tRNA involved in the expression of this unique protein. Specific antibodies against this protein were prepared by synthesizing a 22 amino acid peptide which corresponds to the readthrough portion of the beta-globin readthrough protein, coupling the peptide to KLH protein and injecting the conjugated protein into a sheep. Specific antibodies were produced which were purified and used to isolate the readthrough protein for characterizing the amino acid at the suppression site.		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Dolph L. Hatfield	Research Biologist	LEC NCI
Snorri S. Thorgeirsson	Chief	LEC NCI
Michael Bustin	Research Chemist	LMC NCI

Objectives:

The major goal of this project is to isolate the rabbit beta-globin readthrough protein from rabbit reticulocytes and identify the amino acid at the suppression site.

Specific steps to achieve this goal are: (1) prepare antibodies to the read-through portion of the rabbit beta-globin polypeptide which results from suppression of the corresponding termination codon in rabbit reticulocytes, (2) purify and isolate antibodies specific to this protein, (3) use the purified antibodies to isolate the readthrough protein, and (4) determine the amino acid at the readthrough site.

Methods Employed:

A peptide which is 22 amino acids in length and which corresponds to the portion of rabbit beta-globin polypeptides that results from readthrough was synthesized and coupled to KLH protein by Peninsula Laboratories, Inc. A sheep was immunized by intradermal and intramuscular injection with the coupled protein. The sheep was bled at 3 weeks and at 3-week intervals thereafter. Antibodies were detected by the Elisa technique and were purified using CNBr-activated Sepharose. The purified antibodies were subsequently attached to CNBr-activated Sepharose in order to isolate the readthrough protein.

Protein synthesis was carried out in the presence of rabbit reticulocyte lysates and ³⁵S-methionine and with or without an opal suppressor tRNA. The read-through protein was characterized by polyacrylamide gel electrophoresis and by Western immunoblotting.

Major Findings:

Specific antibodies to the rabbit beta-globin readthrough protein were produced by immunizing a sheep with a peptide corresponding to the readthrough portion of this protein. The antibodies were purified and used to isolate this unique protein from rabbit reticulocytes. The readthrough protein is presently being sequenced near the carboxy terminus in order to identify the amino acid at the suppression site.

The readthrough protein has also been characterized in rabbit reticulocytes. Labeling the protein in reticulocytes with ³⁵S-methionine demonstrates that approximately 0.5% suppression of the beta-globin mRNA naturally occurs in reticulocytes. The presence of the beta-globin readthrough protein in reticulocytes was identified by Western blotting and by enhancement of its synthesis in response to an opal suppressor tRNA.

Z01CP05495-01 LEC

Publications:

None

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05496-01 LEC
PERIOD COVERED February 11, 1986 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Covalent Interactions of Potentially Carcinogenic IQ Derivatives		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: Peter P. Roller	Head, Biopolymer Chemistry Section	LEC NCI
Others: Snorri S. Thorgeirsson	Chief	LEC NCI
Elizabeth G. Snyderwine	Guest Researcher	LEC NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Experimental Carcinogenesis		
SECTION Biopolymer Chemistry Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: <div style="text-align: center;">1.1</div>	PROFESSIONAL: <div style="text-align: center;">1.1</div>	OTHER: <div style="text-align: center;">0</div>
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Three aspects of this project are presently being pursued: (1) Chemical synthesis of potentially carcinogenic derivatives of 2-amino-3-methyl-imidazole-[4,5-f]quinoline (IQ). These derivatives include nitro-IQ, N-hydroxy-IQ, nitroso-IQ, azoxy-IQ, azo-IQ, and acetoxy-acetamide-IQ. Purification of these compounds is achieved through recrystallization and high performance liquid chromatography (HPLC). Structure identification is confirmed by ultraviolet absorbance spectrometry and electron impact mass spectrometry. Synthesis of radiolabeled derivatives is also underway in order to facilitate detection of DNA adducts. (2) Reaction of derivatives with DNA. DNA adducts of IQ derivatives are being chemically synthesized in vitro under various reaction conditions. The extent of the reaction is being tested using various deoxy-nucleotides, deoxynucleoside, and polynucleotides, including calf thymus DNA. Reactivity of specific IQ derivatives, such as N-hydroxy-IQ and acetoxy-acetamide-IQ with deoxynucleotide bases are currently being examined. Adducts are initially identified by HPLC or, if necessary, with the use of radiolabeled precursors. Adducts are further identified by ultraviolet absorbance, spectra and mass spectral analysis. (3) Mutagenicity studies. Ames Salmonella mutagenicity tests are currently underway with IQ derivatives in order to assess the ultimate mutagenic derivatives of IQ. Attempts to correlate the ability of a derivative to covalently bind to DNA with its mutagenicity will be made.</p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Peter P. Roller	Head, Biopolymer Chemistry Section	LEC NCI
Snorri S. Thorgeirsson	Chief	LEC NCI
Elizabeth G. Snyderwine	Guest Researcher	LEC NCI

Objectives:

Food has been considered an important factor for the genesis of cancer in man and animals. 2-amino-3-methyl-imidazole-[4,5-f]quinoline (IQ) is a recently discovered food mutagen which is carcinogenic in rodent models. Its arylamine structure suggests that its mechanism of mutagenicity and carcinogenicity may be a consequence of its metabolism to N-hydroxy-IQ and/or other carcinogenic species. Therefore, the first objective is to chemically synthesize IQ derivatives that are likely to be formed in vivo. Since covalent binding of carcinogens to DNA has been shown to be a critical event in the process of chemical mutagenesis and carcinogenesis, our second objective is to determine if IQ derivatives covalently bind to DNA and to further elucidate the structure of these DNA adducts. The third objective is to pursue the mutagenicity of these derivatives in order to correlate their ability to form specific adducts with their mutagenic potential. Our final objective is to examine, with in vivo animal models, the extent of adduct formation with DNA in target tissues.

Methods Employed:

The principle methods employed are the following: (1) chemical synthesis, (2) HPLC, (3) ultraviolet absorption spectrometry, (4) mass spectral analysis, and (5) Ames Salmonella mutagenicity assays.

Major Findings:

Methods have been developed for the synthesis of many of the derivatives of IQ including nitro-IQ, azoxy-IQ, and the most likely putative carcinogenic derivative, the N-hydroxy-IQ. These compounds have been purified and their structures confirmed by ultraviolet spectrometry and mass spectral analysis. In addition, two HPLC methods have been developed. One method enables us to rapidly monitor the synthesis of the N-OH-IQ from the reduction of the nitro-IQ. With the second HPLC method, we can separate complex mixtures of deoxynucleosides, nucleotides, IQ and the IQ derivatives. The latter HPLC method is currently being used to monitor the formation of adducts between the various nucleosides and the N-OH-IQ.

Publications:

None

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05497-01 LEC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Altered Polypeptide Expression during Mammary Carcinogenesis		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: Peter J. Wirth	Expert	LEC NCI
Others: Peter J. Worland Timothy Benjamin Dolores M. Schwartz Snorri S. Thorgeirsson Unnur Thorgeirsson	Guest Researcher Chemist Biologist Chief Visiting Associate	LEC NCI LEC NCI LEC NCI LEC NCI LP NCI
COOPERATING UNITS (if any) Department of Pathology, University Hospital of Iceland (Valgardur Egilsson)		
LAB/BRANCH Laboratory of Experimental Carcinogenesis		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: <div style="text-align: center;">1.6</div>	PROFESSIONAL: <div style="text-align: center;">1.2</div>	OTHER: <div style="text-align: center;">0.4</div>
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> The project was initiated to identify and characterize tumor-associated protein changes during both human and experimental mammary carcinogenesis. Analysis of polypeptide changes during human mammary carcinogenesis revealed both qualitative and quantitative polypeptide differences. Six cytosolic polypeptides were expressed in all malignant tissues (eight individuals) but not in normal tissue, while one polypeptide (p52; pI 7.40/52 kDa) was not expressed during carcinogenesis. More numerous quantitative changes were also noted. These changes were localized mainly in the pI range 5.8-7.0 and molecular weight ranges of 22-40 kDa. Comparison of polypeptides in this region revealed a general up-regulation of polypeptide expression in malignant tissues as compared to those from normal mammary tissue. Included in this group of polypeptides is one polypeptide, p24 (pI 6.15/24 kDa), which was expressed in greatest concentrations in tissues exhibiting the highest estrogen receptor (ER) content. Expression of p24 was markedly reduced in normal tissue and in malignant tumors possessing low levels of progesterone receptor (PgR)/ER content. This polypeptide (p24) is distinct from any of the major known milk proteins and from the 24K protein of MCF-7 cells, a well-studied differentiation marker in human breast cancer. </p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Peter J. Wirth	Expert	LEC NCI
Peter J. Worland	Guest Researcher	LEC NCI
Timothy Benjamin	Chemist	LEC NCI
Dolores M. Schwartz	Biologist	LEC NCI
Snorri S. Thorgeirsson	Chief	LEC NCI

Objectives:

Mammary carcinogenesis is a multistage process in which genetic and/or epigenetic alterations occur. These alterations become fixed and following appropriate selection pressures are manifest as heritable malignant transformation. In both human and animal carcinogenic studies the growth and development of mammary tumors are under endocrine control and can be manipulated in vitro. In human breast cancer, analysis of cytoplasmic estrogen (ER) and progesterone (PgR) receptor content of human breast tumors is now recognized as an extremely useful clinical tool for the prognosis and evaluation of the sensitivity of tumors in endocrine therapy. Tumors lacking ER respond infrequently to endocrine treatment, whereas 50-60% of those tumors positive for ER and 80% of those which are positive for both ER and PgR receptors are responsive to endocrine therapy. Although these receptor assays are clinically important, numerous inherent problems associated with steroid-receptor binding studies in general have limited their usefulness and have prompted searches for additional biochemical markers of transformation-associated changes in human breast tumors in order to detect, diagnose, and treat this form of cancer as early as possible. The main objectives of this project are to investigate and characterize tumor-associated polypeptide changes in human breast cancer and compare these changes to those observed during chemically-induced [N-nitroso-N-methylurea (NMU)] rat mammary carcinogenesis in an attempt to define and characterize domains of cellular polypeptides which may be involved in the malignant conversion and metastatic capacity of breast tissue.

Methods Employed:

The principal methods employed are: (1) tissue culture techniques, (2) cell separation techniques, (3) histochemical staining; (4) enzyme assays involving radiometric (tritium, carbon-14, sulfur-35 phosphorous-32, and iodine-125) assays, (5) differential centrifugation and chromatographic techniques, (6) autoradiography and fluorography, (7) two-dimensional gel electrophoresis, (8) Western transfer and immunoblot analysis, and (9) computer-assisted quantitation of autoradiograms and silver stained gels.

Major Findings:

(1) In collaboration with Dr. Valgardur Egilsson, Department of Pathology, University Hospital of Iceland, malignant breast biopsy tissue was obtained from eight patients along with normal, nonmalignant tissue from noninvolved sites from the same individuals. The tumors were primarily infiltrating ductal

ANNUAL REPORT OF
THE LABORATORY OF EXPERIMENTAL PATHOLOGY
CHEMICAL AND PHYSICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1985 through September 30, 1986

The Laboratory of Experimental Pathology plans, develops and implements research on the experimental pathology of carcinogenesis, especially concerned with the induction of neoplasia by chemical and physical factors in epithelial tissues, including: (1) development, characterization and evaluation of experimental pathology models of human cancer, such as cancers of the respiratory tract, by in vivo and in vitro carcinogenesis methods; (2) development and characterization of tissue culture systems for quantitative study of the effects of carcinogens alone or in combination; and (3) research on mechanisms of carcinogenesis correlating different levels of biological organization, from whole organisms (human and animal), organs and tissues, to the cellular, subcellular and molecular levels.

General Research Objectives

The main program of investigation in the Laboratory of Experimental Pathology (LEP) is concerned with two correlated problems: (1) the pathogenesis of chemically induced neoplastic disease, particularly in lining epithelia, which are the tissues of origin of most human cancers, studied at sequential levels of biological organization, ranging from human tissues and animal models to organ and cell cultures, and to the biochemical and molecular levels; and (2) the interactions resulting from concurrent effects of different factors in multifactorial carcinogenesis mechanisms, including the role of carcinogens, promoters, oncogenes, growth factors, cellular mediators and certain types of tissue injury.

In order to correlate mechanisms of carcinogenesis, investigated at the cellular and molecular levels, with the corresponding events in animal and human tissues and organs, it is important to connect these different levels of observation and to study the effects of carcinogens in a series of biological systems related to each other in a step-by-step sequence. Such systems include molecular targets, cultured cell systems, organized tissues in culture and in vivo, and finally organs and whole organisms, including not only models of animal pathology but also human pathology. Such an approach requires the development of a range of biological models related to human cancer pathology and particularly to those epithelial target tissues from which most of the major forms of human cancer originate. A great deal of progress has occurred in this direction in the past two decades and experimental animal models have been established, by chemical induction, for most of the major types of human cancer. For many of these models adequate culture systems have been developed for the target tissues and cells, including both animal and human target cells. Pathogenetic mechanisms have been clarified, considerably, through major advances in experimental pathology, cell biology, molecular biology and biochemistry. Work in the LEP has contributed substantially to this progress. The current LEP program represents a logical sequence to these advances.

Current studies are focussed on cell culture systems for rodent and human epithelial cell systems. Emphasis is given to the use of serum-free, possibly chemically defined media. In vivo studies of carcinogenesis by chemical and physical agents focus on multifactorial induction mechanisms of respiratory tract tumors.

I. Cellular Models and Mechanisms

The following main epithelial systems are currently used in the LEP for studies on mechanisms of carcinogenesis:

A. Respiratory epithelia - Cell culture systems for rat and hamster respiratory epithelial in serum-free media have been investigated. These two species offer respiratory carcinogenesis models that differ considerably from each other. Rat respiratory epithelial culture and transformation systems in serum-free media have been established and used for the study of mechanisms of transformation, induced spontaneously, by chemicals and by oncogenes. Stepwise stages of neoplastic transformation were induced by these mechanisms, alone or in combination.

B. Epidermal keratinocytes - The mouse epidermal carcinogenesis model has been widely studied in vivo for decades for its response to full carcinogens and/or promoting agents. Previous work in the LEP led to the development of primary culture methods for mouse keratinocytes in conventional media for the study of differentiation and transformation (Yuspa, S., et al., Transplant. Proc. 12: suppl. 1, 114-122, 1980). Currently, serum-free and nearly chemically defined media have been developed and used for the continuous culture of mouse keratinocytes and for studies on growth, differentiation and transformation. Altered sensitivity to growth factors and inhibitors was demonstrated at higher passage levels.

C. Prostate epithelia - A normal human prostate epithelial cell line and a corresponding prostate carcinoma cell line, established previously (Lechner, J. F., J. Natl. Cancer Inst. 60: 797-801, 1978; Kaighn, M. E., et al., Urology 17: 16-23, 1979) are used for the identification of transforming genes present in the carcinoma line and their role in the transformation of the normal epithelial line. Metastasizing variants of the carcinoma cell line have been characterized.

D. Other epithelial - Liver cell lines were used in bioenergetic studies and other liver cell preparations were used for some studies on carcinogen binding.

E. Nonepithelial cell systems - Selected studies are conducted on nonepithelial cell systems for which appropriate culture conditions are established. (1) BALB/3T3 clone A31-1-1, a mouse embryo cell line, is used for quantitative analysis of carcinogen-induced neoplastic transformation, mutation and DNA damage/repair mechanisms and for studies on chemically induced activation of transforming genes. (2) Chinese hamster ovary (CHO) cells are used for quantitative studies on mutation and DNA damage/repair. (3) NIH/3T3 cell lines are used for transfection assays of DNA-mediated transforming activity.

Development of chemically defined culture conditions for studies of differentiation and transformation in epithelial systems

Methods for chemically induced neoplastic transformation of epithelial cells in culture started to develop in the last decade, but these methods need to be

further extended and more rigorously defined from a quantitative point of view. As new and better defined culture conditions are established for target epithelial cell systems, their response to carcinogens needs to be correlated with the mechanisms of neoplastic transformation investigated at the molecular level. Some of the epithelial systems described above (e.g., mouse keratinocytes, respiratory epithelia) have comparable patterns of response to specific treatments and culture conditions that can lead, on the one hand, to terminal differentiation, senescence and cell death and, on the other hand, to progressive cell growth, anchorage independence and neoplastic transformation. An important condition for studies on these mechanisms is the ability to grow the target epithelia in serum-free, possibly chemically defined culture media, replacing serum with selected additions of hormones and growth factors at optimal concentrations. Current studies investigate factors that control either continuous cell growth or the induction of senescence and terminal differentiation as well as the induction of escape from senescence, yielding continuously growing, preneoplastic and neoplastic transformed cell populations, using mouse keratinocytes and rat tracheal cell lines. Elimination of serum from the culture media in several cells systems has not only disposed of a source of uncontrolled biological variables from batch to batch, but also made it possible to analyze the specific role of individual growth factors and inhibitors in the control of cell growth and transformation.

Mechanism studies with different carcinogens, oncogenes and cofactors

A. Quantitative studies of transformation, mutation, toxicity and DNA damage/repair induced by carcinogens in selected cell systems. Concurrent induction of these different biological responses in selected cell systems has been quantitatively investigated under various experimental protocols that revealed patterns of dissociation of the responses. Of particular interest was the finding of a marked temporal dissociation between mutation and transformation responses obtained by varying the exposure durations to alkylating agents in BALB/3T3 cells, resulting in an early maximal induction of ouabain resistance (oua^r) mutations and single strand breaks, whereas a much longer exposure duration was required for the maximal induction of transformation and of cytotoxicity. CHO cells showed the same early response for oua^r mutations but longer induction kinetics for 6-thioguanine-resistant (6-TG^r) mutations.

The establishment of quantitative assays for mouse keratinocytes and rat tracheal epithelial cells has made it possible to investigate spontaneous and induced transformation mechanisms, quantitatively, in these epithelial systems.

B. Identification and characterization of genes involved in carcinogenesis. Current work is devoted to (a) the identification of three separate transforming DNAs obtained from BALB/3T3 cells transformed by benzo[a]pyrene (BP) and having different sensitivity to restriction enzymes, (b) the role of single or multiple oncogenes transfected in normal and preneoplastic rat tracheal epithelial cells, and (c) the identification of oncogenes in a human prostatic carcinoma cell line.

C. Investigation of biochemical mechanisms and markers in carcinogenesis models. The following areas of investigation have been pursued with biochemical and biophysical methods relevant to carcinogenesis models. (a) Methods recently developed in the Laboratory of Pathology, DCBD, NCI, for the detection of collagenase IV activity have been selected for studies on the timing of induction of

this enzyme during neoplastic transformation. Activation of this enzyme, which digests basal membrane collagen, is needed for cell invasiveness. These studies are designed to test whether this enzyme can be used as a marker for the acquisition of established malignant transformation in various cell types. (b) The binding of carcinogens to specific DNA regions was investigated; high binding levels were found in DNAase I-hypersensitive regions. (c) Studies of benzo[a]-pyrene metabolism and DNA binding were conducted comparatively in various segments of the hamster respiratory tract in vivo and in vitro.

D. Bioenergetic studies of transformed cells. Bioenergetic pathways and the characteristics of the lactic dehydrogenase (LDH) system were studied in non-transformed and in neoplastic rat liver cells.

II. In vivo studies on multifactorial mechanisms of respiratory carcinogenesis

In vivo studies on respiratory carcinogenesis are continuing a long line of research in this field by the present investigators. The hamster respiratory carcinogenesis model (Saffiotti, U., et al., Cancer Res. 28: 104-124, 1968) has become well established as closely resembling the pathogenesis of human bronchogenic carcinoma and continues to be used for in vivo studies of cell differentiation and tumor induction by various carcinogens and cofactors. Extensive in vivo studies of respiratory tract carcinogenesis in both hamsters and rats are under way in the Laboratory. Induced tumors provide a source for the culture and characterization of malignant cells and for studies on the activation of oncogenes. Two strains of hamsters (inbred and outbred) and a strain of rats are used both for long-term experiments on induced tumor pathology and for serial sacrifice experiments designed to study the stages of tumor pathogenesis, the cells of origin and the concomitant pathology following treatment protocols with different carcinogens and cofactors. A series of experiments has been completed on the role of combined treatments with topical and systemic chemical carcinogens, with or without physical factors such as microtrauma and inorganic particulates, and/or other toxic chemicals. The kinetics of the proliferative response of the target epithelium is studied to characterize the cells of origin of the hyperplastic, metaplastic and neoplastic reactions of the respiratory epithelium. Different segments of the respiratory tract are studied for their different responses to various experimental conditions, including the nasal epithelium, the trachea, the bronchi and the peripheral airways.

A new research approach has been developed on the basis of previous and current findings linking the granulomatous fibrogenic reaction induced by crystalline silica in rat lungs with an early and sustained hyperplastic proliferation of the peripheral airway epithelia and with the eventual induction of pulmonary carcinomas. The hamster provides a negative model, lacking both the fibrogenic and the carcinogenic responses to silica.

In summary, LEP research is addressed to the pathogenesis of epithelial neoplasia, through the elucidation of mechanisms that induce neoplastic transformation when concurrently or sequentially activated. These mechanisms are studied in animal models correlated with the main epithelial tissues of origin of human cancers in vivo, in the corresponding cell culture models, maintained by optimally defined culture conditions, and finally at the biochemical and molecular level.

OFFICE OF THE CHIEF

(1) Provides overall scientific direction and administrative coordination to the Laboratory's intramural research program and its supporting resources; (2) participates in research projects in all components of the Laboratory and provides collaborative research coordination of staff activities and resources; (3) conducts bibliographic research and data analysis; and (4) conducts research on carcinogenesis mechanisms and quantitative studies on the interactive effects of combined exposures to different carcinogens and cofactors, using in vivo and in vitro systems established in the Laboratory.

Investigators assigned to this Office conduct basic research on mechanisms of neoplastic transformation of cells in culture, on biochemical mechanisms and on the molecular characterization of oncogenes involved in chemical carcinogenesis.

Projects are under way in the following areas:

Quantitative studies on the concurrent induction of cytotoxicity, DNA damage and repair, mutation and transformation.

The induction kinetics of the following different genotoxic lesions were studied concurrently: cytotoxicity, ouabain resistance (oua^r) mutations, morphological neoplastic transformation, DNA damage and repair as measured by alkaline elution and removal of alkylated DNA adducts as measured by HPLC. The relative levels of response for these biological end points were determined after treatment with the alkylating agents, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and N-ethyl-nitrosourea (ENU), for varying exposure periods. The short exposure time (5 min for ENU) required for maximal induction of oua^r mutations and single-strand breaks was found to be similar in BALB/3T3 clone A31-1-1 cells that repair O⁶-alkylguanine and for CHO cells that are unable to repair this lesion. Maximal transformation of BALB/3T3 cells by ENU required 45-60 min for exposure and maximal induction of 6-thioguanine resistant (6-TG^r) mutations in CHO cells also required long exposure times. In CHO cells exposed to ENU, combined treatment with the inhibitors of excision repair, 1-beta-D-arabinofuranosylcytosine (ara-C) and hydroxyurea, allowed DNA damage, measured as single strand breaks, to increase for at least 30 minutes of exposure. The induction kinetics of the observed responses appeared to be determined by factors other than the repair kinetics of individual alkylated bases.

The induction of type IV collagenolytic activity was investigated as a marker of the acquisition of invasiveness, the most advanced malignant phenotype. Marked degradation of labelled type IV collagen substrate was induced by a BALB/3T3 cell line transformed by arsenic. The degradation pattern differed from those reported with other cell types; this enzymatic activity is being further studied.

Identification, characterization and cloning of oncogenes in chemically transformed cells or related systems. The mechanisms whereby different genes control carcinogenesis induced by chemical and physical agents are investigated in the cellular systems under study in the Laboratory. Methods employed in these investigations include: DNA transfection followed by selection of specific phenotypes; gene cloning using the sib selection protocol and library screening with specific probes; restriction mapping; subcloning and sequencing of cloned genes; Southern and Northern transfer techniques; and treatment of nuclei with DNAase I to locate DNA-carcinogen adducts and to probe the chromosomal structure of new transforming genes. The following studies were conducted in order to identify, characterize,

and clone those genes that drive the development of neoplasia and whose malignant potential results from changes caused by chemical carcinogens. (1) To identify transforming genes activated by benzo[a]pyrene (BP), DNA from three BALB/3T3 cell lines transformed by BP was analyzed by DNA transfer and focus formation in the NIH/3T3 system. All three tested lines showed transforming activity that differed from each other and from the ras oncogenes by restriction endonuclease sensitivity and MspI mapping. These possibly new transforming genes are now being cloned by the sib selection protocol from Charon 4A phage genomic libraries. Several cycles of selections have been carried out with oncogene activity and the positive pools show no presence of the ras oncogenes. (2) The mechanism by which chemical carcinogens may activate proto-oncogenes was explored by analyzing the distribution of carcinogen adducts on different parts of the genome. BP adducts, formed in vivo in hamster liver cells, were preferentially located in DNAase I hypersensitive regions of the genome and were rapidly removed by repair processes, while persisting adducts remained in other parts of the genome. In target hamster liver cells, the Ha-ras proto-oncogene was found to be present in a transcriptionally active form. (3) Human homologs of pro genes were isolated from the human nasopharyngeal carcinoma cell line CNE2, and characterized.

Bioenergetic pathways in transformed epithelial cells. These studies pertain to the development of aerobic glycolysis in cancer cells, using current biochemical methods to identify modifications in lactate dehydrogenase (LDH, E.C. 1.1.1.27), cytoskeletal complexes and mitochondria. Studies with rat liver cells showed that the steady-state activity of LDH has a ratio of 1:4 between control and chemically-transformed neoplastic cell lines. Specific isozymes associated with either control or neoplastic liver cell lines were determined. Different ratios of LDH-4 and -5 were identified when control liver cell lines in passages up to 21 were compared with neoplastic cell lines. As the passage number increased, control cells showed alterations in the ratio of enzyme activity relative to neoplastic cells. The ratio of LDH-4 and -5 also shifted to 1:1.

RESPIRATORY CARCINOGENESIS SECTION

(1) Conducts research on the pathogenesis of cancers in the respiratory tract and on their induction by carcinogens, alone or in combination, using animal models closely related to human pathology and corresponding in vitro systems; (2) investigates the carcinogenic effects of chemical and physical agents on the respiratory tract, their quantitative aspects and their pathogenetic mechanisms; (3) studies mechanisms of cell differentiation and carcinogenesis in respiratory and related epithelia; and (4) provides pathology expertise, resources and collaboration to other components of the Laboratory in the study of epithelial carcinogenesis.

The research activities of this Section are devoted to the characterization of respiratory carcinogenesis responses in appropriate model systems in vivo and in vitro and to the elucidation of mechanisms of epithelial carcinogenesis by chemical and physical factors, alone or in combination. The programs are closely correlated with those of the other LEP components to which this Section also provides pathology research expertise for in vivo animal carcinogenesis studies.

Projects are under way in the following areas:

Respiratory carcinogenesis by chemical and physical factors. The induction of cancers from the epithelia of the different segments of the respiratory tract in

animal models, by multifactorial mechanisms, is studied by treatment with combinations of chemical, physical and biological factors. Age at beginning of treatment was found to influence the induction of respiratory tumors by diethylnitrosamine in a segment-specific manner. In hamsters treated from birth, there is an increased incidence and decreased latency of nasal cancers; no significant age-related effect was found on the induction of tracheal or lung neoplasms. Concurrent intraperitoneal injection of dimethylsulfoxide increased the incidence and severity and decreased the latency of respiratory tumors induced by intratracheal administration of suspensions of benzo[a]pyrene (BP) combined with ferric oxide (Fe_2O_3). A complex multifactorial experiment of respiratory carcinogenesis in hamsters has been completed and the results are under analysis; preliminary results show that a single dose of N-methyl-N-nitrosourea (MNU) 2 weeks prior to a series of BP/ Fe_2O_3 administrations markedly enhances laryngeal and bronchial carcinogenesis relative to exposure to either carcinogen alone. The single instillation of MNU alone also induced a distant carcinogenic response (pancreas ductular carcinomas and adrenal cortical carcinomas). Localized injury to the trachea characterized by increased epithelial mitotic activity, enhances the carcinogenic effect of BP/ Fe_2O_3 not only in the trachea, but also in the bronchi. Previous exposure to BP/ Fe_2O_3 increases DNA binding of BP in all exposed segments of the respiratory tract, with the trachea showing the highest and the most transient binding levels.

Role of silica-induced granulomatous fibrogenic inflammation in carcinogenesis. Silica-induced pulmonary granulomatous and fibrogenic cell reactions and cellular mediators of inflammation are studied for their pathogenetic relationship in conjunction with long-term carcinogenesis studies of different forms of silica (quartz, tridymite, cristobalite). Marked epithelial proliferative hyperplasia was described in peripheral airways adjacent to granulomatous reactions. Species specificity is investigated comparatively in rats, hamsters and mice. The working hypothesis has been proposed that the intense granulomatous reaction induced by silica (macrophages, fibroblasts, lymphocytes, plasmacells, mastcells, and polymorphonuclear leukocytes), previously studied in detail, generates a continuous release of cellular mediators of inflammation which act to stimulate the adjacent respiratory epithelium. These mediators include reactive oxygen (already known to be involved in epithelial cell stimulation and promotion), as well as many cytokines (e.g., interleukin-1), enzymes, and several other factors that have not been adequately studied for their effects on target epithelia. This new research approach has far-reaching implications for a fresh re-evaluation--by modern methods and in specific terms--of the relationship between certain chronic inflammatory reactions and carcinogenesis.

TISSUE CULTURE SECTION

(1) Conducts research on cell culture systems for the characterization and quantitative study of neoplastic transformation induced by chemical and physical carcinogens; (2) develops and characterizes organ and cell culture systems for carcinogenesis studies, especially those derived from epithelia known for their susceptibility to carcinogens in vivo, such as the respiratory tract epithelium, (3) conducts research on mutagenesis, neoplastic transformation, differentiation, and on their expression mechanisms and relationships; and (4) provides expertise, resources and collaboration on tissue culture methods for the entire Laboratory.

The objective of the research program of the Tissue Culture Section is to understand how carcinogenic agents alter the control of growth and differentiation at both the cellular and molecular levels and how cells progress to neoplasia through

a sequence of genomic and/or epigenetic alterations. Emphasis is placed on studies of epithelial cells derived from tissues representing major organ sites relevant to human cancer. Cell systems currently under study include mouse epidermal keratinocytes, rat and hamster respiratory epithelial cells and human prostatic epithelial cells. In addition, mouse embryo cell lines (BALB/3T3) and NIH/3T3 are used for studies on transformation mechanisms (See also: Office of the Chief).

The following projects are under way:

Growth of mouse keratinocytes in serum-free medium. An improved serum-free medium (LEP/MK2) has been developed for mouse keratinocytes (MK) and found to support continuous growth for at least 75 subcultures (more than 200 doublings). The established line has a reduced requirement for the factors in LEP/MK2. In addition, the responses to serum components decrease with time in culture. At high passage levels, sensitivity to growth inhibition by serum and by TGF-B was found to decrease with time, but was not abolished. MK cells in culture rapidly lose their normal karyotype. By passage 4, they are essentially tetraploid with random gains and losses of individual chromosomes. These rapid chromosomal alterations may form a basis for the observed altered response to exogenous factors. The MK system is used to develop a reliable selective assay for epithelial transformation and to investigate mechanisms of transformation by chemical carcinogens and oncogenes.

Cellular and molecular stages of carcinogenesis in respiratory epithelia. For rat tracheal epithelial RTE cells, an improved serum-free medium (LEP/RTE 2) was developed which supports clonal growth of primary RTE cells. The growth promoting activity of bovine pituitary extract, a critical component of LEP/RTE 2 medium, was found to be acid sensitive, heat stable, trypsin insensitive and had a molecular weight >5000. When the induction of preneoplastic RTE variants by the carcinogens, MNNG and benzo[a]pyrene-diol-epoxide (BPDE) in cells plated in serum-containing medium on feeder cells or in serum-free medium was compared, MNNG was considerably more cytotoxic and potent as a transforming agent in serum-free than in serum-containing medium. The cytotoxic and transforming activities of BPDE were equivalent under both conditions. Development of serum-free media for both of these systems simplifies studies of altered growth control in carcinogenesis.

Changes, at the cellular and molecular levels, responsible for the development of preneoplastic and neoplastic variants of RTE cells, were investigated. To identify cells having critical preneoplastic changes, comparative transformation experiments were conducted in which independent populations of preneoplastic RTE cells were treated with chemical carcinogen MNNG or with cloned oncogene-containing DNAs from Harvey murine sarcoma virus (HaMSV, ras), polyoma virus, and MC29 virus (myc). Differential responses of various cell lines to these treatments showed evidence of differences in their preneoplastic potential. Using the hypothesis that differential responses of "preneoplastic" cell lines to carcinogens or oncogenic DNAs have a genetic basis, efforts will be directed towards the molecular cloning of the genes responsible for the conversion of preneoplastic to neoplastic cells. These studies require the development of assays suitable for the detection of these genes. Preneoplastic RTE cells can be used cells for identification of RTE cell tumor genes. This assay for tumor genes provides an intraspecific system in which the genes can be identified in cells that are genetically related to the cells in which the gene was originally active. Transfections of normal RTE cells with cloned oncogene-containing DNAs

were also investigated and showed induction of escape from senescence but not full neoplastic transformation, whereas transfection into chemically induced preneoplastic variants of RTE cells induced neoplastic phenotypes in all lines treated with polyoma and in some of those treated with ras.

Cellular and molecular studies in normal and neoplastic human prostatic cells.
A major goal of this project is to detect and clone a transforming gene from the human prostatic cancer cell line, PC-3. Transforming activity has been found in PC-3 genomic DNA by the NIH/3T3 cell focus assay. Using the nude mouse tumorigenicity assay in conjunction with drug selection of NIH/3T3 cotransfected cells, the presence of an activated oncogene was confirmed. This unidentified oncogene, which does not appear to be a member of the ras family, is being cloned by sib selection using human repeated sequences as a probe. Preliminary results suggest the possible presence of suppressor DNA sequences that inhibit tumorigenicity of certain NIH/3T3 cells. Isolation of this suppressor activity also has been undertaken by sib selection. Autocrine activities of PC-3 and its highly metastatic variants are being investigated in collaboration with D. Sirbasku (University of Texas). An ascites line (PC-3asc) was isolated directly in serum-free medium from a tumor obtained by inoculation of PC-3/mA2 in the nude mouse in collaboration with J. Kozlowski (Northwestern University). The PS-3asc line was found to produce an autogenous factor that stimulated its own growth at low cellular inocula. In addition, preliminary data indicate that medium conditioned by PC-3asc cells has TGF activity in normal rat kidney (NRK) cells in soft agarose. This putative TGF is being isolated and will be further purified by standard biochemical procedures. Karyotypic analysis has shown that the PC-3asc line has many features in common with the parental line, PC-3/mA2, except for a few marker chromosomes with homogeneously staining regions and numerous minute and double minute chromosomes.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP04491-10 LEP
PERIOD COVERED October 1, 1985 through September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Quantitative Studies on Concurrent Factors in Neoplastic Transformation		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	U. Saffiotti Chief	LEP NCI
Others:	M. E. Kaighn Expert S. Garbisa Guest Researcher	LEP NCI LP NCI
COOPERATING UNITS (if any) Laboratory of Toxicology, Istituto Superiore di Sanita', Rome, Italy (M. Bignami, E. Dogliotti).		
LAB/BRANCH Laboratory of Experimental Pathology		
SECTION Office of the Chief		
INSTITUTE AND LOCATION NCI, NIH, Frederick, Maryland 21701-1013		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.9	0.4	0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The induction kinetics of different genotoxic lesions were studied concurrently with the following assays: cytotoxicity, ouabain resistance (oua-r) mutations, morphological neoplastic transformation, DNA damage and repair as measured by alkaline elution, and removal of alkylated DNA adducts as measured by HPLC. The relative levels of response for these biological end points were determined after treatment with the alkylating agents, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and N-ethylnitrosourea (ENU), for varying exposure periods. The short exposure time (5 min for ENU) required for maximal induction of oua-r mutations and single-strand breaks was found to be similar in BALB/3T3 clone A31-1-1 cells that repair O6-alkylguanine and for CHO cells that are unable to repair this lesion. Maximal transformation of BALB/3T3 cells by ENU required 45-60 min for exposure, and maximal induction of 6-thioguanine-resistant (6-TG-r) mutations in CHO cells also required long exposure times. In CHO cells exposed to ENU, combined treatment with the inhibitors of excision repair 1-beta-D-arabinofuranosylcytosine (ara-C) and hydroxyurea allowed DNA damage, measured as single strand breaks, to increase for at least 30 minutes of exposure. The induction kinetics of the observed responses appeared to be determined by factors other than the repair kinetics of individual alkylated bases.</p> <p>The induction of type IV collagenolytic activity was investigated as a marker of the acquisition of invasiveness, the most advanced malignant phenotype. Marked degradation of labelled type IV collagen substrate was induced by a BALB/3T3 cell line transformed by arsenic. The degradation pattern differed from those reported with other cell types.</p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

U. Saffiotti	Chief	LEP	NCI
M. E. Kaighn	Expert	LEP	NCI
S. Garbisa	Guest Researcher	LP	NCI

Objectives:

(a) To study mammalian cell culture systems for concurrent induction of cytotoxicity, DNA damage and repair, mutagenicity and neoplastic transformation in order to define the interrelationships of these end points and their induction kinetics in response to different exposure durations to carcinogens. (b) To investigate the induction of invasiveness as a marker of advanced transformation.

Methods Employed:

The BALB/3T3 clone A31-1-1 mouse embryo cell line was used under test conditions previously standardized in this laboratory for transformation assays with different carcinogens and for the induction of ouabain resistance (oua^r). Analogous studies were also conducted in Chinese hamster ovary (CHO) cells for oua^r mutations and 6-thioguanine-resistant (6-TG^r) mutations. DNA damage and repair were determined by alkaline elution analysis and removal of alkylated DNA adducts was measured by HPLC analysis. Duration of exposure to alkylating agents was studied in time-course experiments for toxicity, mutation, transformation, DNA damage and repair. Two alkylating agents were used for treatments: N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and N-ethylnitrosourea (ENU).

Degradation of labelled type IV collagen substrate was measured in control and chemically transformed BALB/3T3 cell lines, and the collagen degradation pattern was examined by gel electrophoresis.

Major Findings:

Duration of exposure to alkylating agents was found to be an important parameter that results in a marked temporal dissociation of different biological responses in both BALB/3T3 and CHO cells. As reported last year, varying the duration of exposure to MNNG and to ENU in BALB/3T3 cells revealed that maximal induction of oua^r mutations and of DNA damage as single strand breaks (ssb) were obtained with exposures much shorter than the half-life of the alkylating agent, whereas maximal frequency of neoplastic transformation required exposures three to four times longer than the half-life.

Similar studies were conducted in CHO cells, which were confirmed to lack the ability to repair O⁶-alkylguanine, in comparison with the BALB/3T3 cells which showed fairly rapid repair of this lesion. Both cell types showed the same early peak for maximal induction of oua^r mutations. In contrast, 6-TG^r mutations in CHO cells showed slower induction kinetics, compatible with the calculated curve for exponential decay of ENU in time.

The underlying mechanisms were investigated in both cell types by analysis of repair of DNA ssb and of individual alkylated bases. When CHO cells were exposed to ENU concurrently with the inhibitors of excision repair, ara-C and hydroxyurea, DNA damage no longer peaked after 5 min, but continued to increase for at least 30 min. The repair kinetics of the individual alkylated bases, so far examined by HPLC analysis, did not provide a sufficient basis for the interpretation of the different induction kinetics of mutations and transformation in these cell systems.

Type IV collagen degradation by control and chemically transformed BALB/3T3 cells was determined by measuring either the release by cultured cells of degraded type IV collagen from wells coated with labelled collagen or by measuring collagen degradation by conditioned media obtained from cultures of test cells. The latter method gave more reproducible results. The highest activity was found in a BALB/3T3 line transformed by arsenic. Gel electrophoretic analysis of the type IV collagen degradation products showed that only one of the two alpha-chains of collagen was degraded, whereas previous studies with other cell types showed degradation of both chains. This study continues in collaboration with the Laboratory of Pathology, Division of Cancer Biology and Diagnosis, NCI.

Publications:

Saffiotti, U., Bignami, M. and Kaighn, M. E.: Parameters affecting the relationships among cytotoxic, genotoxic, mutational and transformational responses in BALB/3T3 cells. In Barrett, J. C. and Tennant, R. W. (Eds.): Mammalian Cell Transformation. Raven Press, New York, 1985, pp. 139-151.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP04493-08 LEP
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Bioenergetic Pathways in Chemically-Transformed Epithelial Cells		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: A. E. Kaplan Research Chemist LEP NCI		
COOPERATING UNITS (if any) Laboratory of Applied Studies, Division of Computer Research and Technology, NIH, Bethesda, MD (B. Bunow); Department of Microbiology, Harvard Medical School, Boston, MA (H. Amos); Program Resources, Inc., Frederick, MD (R. L. Brown)		
LAB/BRANCH Laboratory of Experimental Pathology		
SECTION Office of the Chief		
INSTITUTE AND LOCATION NCI, NIH, Frederick, Maryland 21701-1013		
TOTAL MAN-YEARS: <div style="text-align: center; font-weight: bold;">2.2</div>	PROFESSIONAL: <div style="text-align: center; font-weight: bold;">1.0</div>	OTHER: <div style="text-align: center; font-weight: bold;">1.2</div>
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Studies of routes of energy losses from neoplastic cells continued using a model consisting of a control rat hepatocyte line and its N-nitroso-N-methylurea-transformed counterpart. Studies of energy loss focused on the enzyme, lactic dehydrogenase (LDH, E.C. 1.1.1.27), because it is known to produce lactic acid in larger quantities in neoplastic cells and to export it into the medium, representing an energy loss. Eight subtypes of LDH were identified with isoelectric points between pH 5.96 and 9.46. Four acidic proteins below pH 7 are more prominent in LDH from the control cell and are believed to be phosphorylated proteins on the basis of previous results. In neoplastic cells, 35% of the glucose taken up for cell growth is converted to lactic acid, representing a sizeable loss of energy compared with the 8.5% converted to lactic acid by the control cell. Earlier studies of LDH behavior suggested this difference.</p> <p>Structural studies on cells grown on microspheres and on flat surfaces show the ability of the neoplastic cell to grow attached to a more limited surface area than the control cell, compared within the same time periods. Neoplastic cells continue to grow on the spherical surface, whereas control cells grow beyond the area and spread on the flat surface of the flask. Study of the cytoskeletal proteins, actin, cytokeratin and tubulin, showed that, whereas tubulin appears to be the same in both cell lines, cytokeratin is reduced and actin is markedly reduced in the neoplastic cell, especially in the region of the plasma membrane, suggesting that morphological changes in the membrane may relate to the loss of adequate cytoskeletal attachments.</p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

A. E. Kaplan Research Chemist LEP NCI

Objectives:

The objectives of this project are to characterize modifications occurring with neoplastic transformation which correlate energy loss and its structural changes. In this study, a control and its chemically transformed neoplastic rat hepatocyte line are used as a model experimental system.

1. To characterize molecular modifications in lactate dehydrogenase (LDH, E.C. 1.1.1.27), which represents an important gate for energy loss in the tumor cell, and more specifically, to determine the pI values of individual LDH proteins; to compare protein structures of post-translationally modified LDH proteins; to compare, by amino acid analysis, extracts from the two cell lines as well as the proteins within each extract for similarities and differences of denatured LDH proteins; to determine the amount of lactic acid exported by the two cell types in order to calculate how much energy is lost from each, and to gain an understanding of the contribution that this enzyme step may represent in the energy loss from a cancer patient.
2. To complete studies by scanning electron microscopy of the growth of cells on microspheres in order to identify different growth patterns with transformation.
3. To complete studies of cytoskeletal proteins and their changes with neoplastic transformation which may contribute to modifications in structure and in anchorage dependence during growth.

Methods Employed:

1. Standard biochemical techniques are used to extract and stabilize LDH from control and neoplastic cell lines of both epithelial and fibroblastic origin. LDH isozymes are identified by gel electrophoresis and pI values are obtained by the isoelectric focusing and Immobiline Electrophoresis methods, which are based on buffered gradients.
2. Scanning electron microscopy is used in studies of growth in microspheres.
3. Fluorescent antibody techniques are used to identify tubulin, cytokeratin, and actin complexes in cells to determine differences between control and neoplastic epithelial cells. Intracellular pH is maintained at neutrality in both cell lines to prevent aggregation of the cytoskeletal proteins.

Major Findings:

1. LDH, identified as two isozymes, LDH-4 and -5, in previous reports, is shown to be made up of eight enzymatically reactive proteins when separated by the Immobiline Electrophoresis method. These LDH proteins migrate to isoelectric

points between pH 5.95 and 9.46. The eight proteins are not equally represented in the extracts from control vs. neoplastic cell lines. The neoplastic cell line consistently shows far more activity among the alkaline LDH types. The alkaline LDH types appear to form LDH-5 in traditional electrophoretic separations, and this isozyme has always been strongly reactive in extracts from the neoplastic cell line. There is also evidence that the acidic LDH proteins are decreased in the neoplastic cell extract.

Among the acidic LDH subtypes observed, a weakly reactive triplet of enzyme bands is observed between pH 6.64 and 6.71 and a single enzyme band at pH. 5.95. From previous experiments in which LDH-4 was altered by exposure to alkaline phosphatase, it appears that the most acid proteins may represent phosphorylated types of LDH. The strongly reactive enzyme band at pH 7.09 appears to be different in posttranslational modification because it persists in both cell lines, irrespective of alterations in the acidic LDH triplet group between pH 6.64 and 6.71.

2. Comparison of lactic acid exported by the two cell lines reveals that the control cell converts 8.5% of the glucose used for growth to this acid, but the neoplastic cell converts 35% of the glucose to lactic acid--far more than was expected. This suggests that the altered kinetic behavior of LDH, discussed in earlier reports, acts as a major site of energy loss in neoplastic cells.

3. Scanning electron microscopy studies of growth of the two cell lines on microspheres is now completed and we have observed that the control cells retain the need to grow with contact areas which do not decrease. This can be observed by the tendency of these cells to begin to grow on the flat surface of the flask when they can no longer find adequate space on the spherical surface. Under the same growth/time conditions, the neoplastic cells continue to adhere to the spherical surface because they can reduce their needs for anchorage dependence during growth. These observations suggest that growth on microspheres can serve not only to grow larger numbers of cells more rapidly, but also as a simple method to observe changes in surface anchorage requirements of cells undergoing neoplastic transformation.

4. Maintaining neutral intracellular pH before fixing cells for immunofluorescent analysis of actin, cytokeratin and tubulin, showed that the first two cytoskeletal proteins are diminished in the neoplastic cell but the third remains constant. Actin, especially, is very sparse in this neoplastic hepatocyte line and this may explain subtle changes in the appearance of this cell at the plasma membrane during different stages of growth compared with the control hepatocyte line.

Publications:

Kaplan, A. E. and Bunow, M. R.: Spectrophotometric determination of intracellular pH with cultured rat liver epithelial cells. J. Histochem. and Cytochem. 34: 749-752, 1986.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05265-05 LEP
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Effects of Chemical Carcinogens on Transforming DNA Sequences and Expression		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	U. Saffiotti	Chief LEP NCI
Others:	M. E. Kaighn	Expert LEP NCI
COOPERATING UNITS (if any) Laboratory of Central Nervous System Studies, National Institute of Neurological and Communicative Disorders and Stroke, NIH (D. Y. Goldgaber); Laboratory of Oral Medicine, National Institute of Dental Research, NIH (M. I. Lerman).		
LAB/BRANCH Laboratory of Experimental Pathology		
SECTION Office of the Chief		
INSTITUTE AND LOCATION NCI, NIH, Frederick, Maryland 21701-1013		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.6	0.5	1.1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> The aim of this project is to identify, characterize, and clone those genes that drive the development of neoplasia and whose malignant potential results from changes caused by chemical carcinogens. (1) DNA from three BALB/3T3 cell lines transformed by benzo[a]pyrene (BP) was analyzed by DNA transfer and focus formation in the NIH/3T3 system. All three tested lines showed transforming activity that differed from each other and from the <u>ras</u> oncogenes by restriction endonuclease sensitivity and MspI mapping. These possibly new transforming genes are now being cloned by the sib selection protocol from Charon 4A phage genomic libraries. Several cycles of selections have been carried out with oncogene activity and the positive pools show no presence of the <u>ras</u> oncogenes. (2) The mechanism by which chemical carcinogens may activate proto-oncogenes was explored by analyzing the distribution of carcinogen adducts on different parts of the genome. BP adducts, formed in vivo in hamster liver cells were preferentially located in DNAase I hypersensitive regions of the genome and were rapidly removed by repair processes, while persisting adducts remained in other parts of the genome. By this approach, the Ha-<u>ras</u> proto-oncogene was found to be present in a transcriptionally active form in target hamster liver cells. (3) Human homologs of <u>pro</u> genes were isolated from the human nasopharyngeal carcinoma cell line CNE2, and were characterized. </p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

U. Saffiotti	Chief	LEP	NCI
M. E. Kaighn	Expert	LEP	NCI

Objectives:

The overall objective of this project is to identify, characterize, and clone cancer genes that drive the development of neoplasia and whose malignant potential results from changes caused by chemical carcinogens. The immediate objectives are: (a) to determine the molecular structure of the transforming genes activated in BALB/3T3 cells transformed by benzo[a]pyrene (BP), (b) to define the role of DNAase I hypersensitive sites in DNA in carcinogen binding and oncogene activation, (c) to characterize the human homolog of mouse pro-genes in nasopharyngeal carcinoma.

Methods Employed:

(a) Transformation was induced with different chemical carcinogens in the mouse embryo cell line, BALB/3T3 clone A31-1-1. The DNA of the transformed cells was analyzed for transforming activity by transfection into NIH/3T3 cells and used as a source for the cloning of activated oncogenes by established and novel approaches.

(b) Brief treatment of nuclei with low concentrations of DNAase I has been used to define DNAase I hypersensitive regions in the chromosome. These DNAase I hypersensitive regions, when mapped, occur in the putative regulatory regions of actively expressed genes. Carcinogen treatment in vivo or in vitro, followed by DNAase I treatment of the nuclei, was used to determine whether or not carcinogens preferentially bind to DNAase I hypersensitive regions. Labeled probes of known oncogenes were hybridized to blots of genomic DNA to study the effects of carcinogen and DNAase I treatments at the gene level.

(c) Human homologs of the mouse pro genes were investigated using a phage library of nasopharyngeal carcinoma DNA screened by a mouse probe.

Major Findings:

Identification of carcinogen-activated oncogenes in BALB/3T3 cells. In order to investigate whether the same or different transforming sequences were induced by the same carcinogen, the three most active BALB/3T3 lines transformed by BP were studied after digestion with the restriction endonucleases, EcoRI, HindIII, BamHI and XbaI. Different patterns of sensitivity to endonuclease activity were obtained and confirmed for each of the three tested lines, showing that different patterns of sensitivity can be induced by the same carcinogen in the same target cells. In these cell lines, hybridization with probes containing Ha-ras, Ki-ras and N-ras oncogenes repeatedly failed to show any mutation, rearrangement or amplification of the ras gene. To clone these genes, Charon 4A

phage genomic libraries were constructed, and the transforming genes are being isolated by sib selection using the NIH/3T3 focus assay. Several cycles of selection gave positive pools for the presence of the transforming gene; they were all negative with the ras oncogene probes.

Role of DNAase I hypersensitive regions in carcinogenesis. In vivo experiments with hamsters showed that the formation of DNA-BP adducts reaches a maximum in the liver within 30 to 60 minutes after injection of [³H] BP into the portal vein. Of these adducts, 70-80% appear to be in DNAase I hypersensitive regions. The state of the Ha-ras locus in hamster liver cells was studied in the same system. The locus is represented in the normal liver by two alleles of 19 kb and 2 kb in size, and the 19 kb gene contains a hypersensitive site. The state of the locus was examined following a single in vivo intraportal treatment with BP. Ten min after BP injection, the hypersensitive region of the 19 kb gene appears to contain a double strand cut, probably introduced by the repair enzymes. Also, the 19 kb gene is more sensitive to DNAase in BP-treated hamster liver as compared to normal liver and persists during the phase of rapid binding and repair. These preliminary observations, using in vivo protocols, show that oncogenes can be rapidly damaged by chemical carcinogens and rapidly repaired in living cells.

Human homologs of mouse pro genes. The ability to confer promoter-sensitive phenotypes on JB6 test cells was investigated for several normal and tumor DNAs. Of these, only DNA from a human nasopharyngeal carcinoma cell line showed this activity. One line was chosen to isolate the human homolog of the mouse genes; only the pro-1 homolog was found to be active in the JB6 test system.

Publications:

Lerman, M. I., Hegamyer, G. A. and Colburn, N. H.: Cloning and characterization of putative genes that specify sensitivity to neoplastic transformation by tumor promoters. Int. J. Cancer 37: 293-302, 1986.

Lerman, M. I. and Colburn, N. H.: Pro genes: a novel class of genes that specify sensitivity to induction of neoplastic transformation by tumor promoters. In Cooper, G. M. (Ed.): Viral and Cellular Oncogenes. Boston, Martinus Nijhoff Publishing. (In Press)

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05274-05 LEP
PERIOD COVERED October 1, 1985 through September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Respiratory Carcinogenesis by Chemical and Physical Factors		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	U. Saffiotti	Chief LEP NCI
Others:	S. F. Stinson	Biologist LEP NCI
COOPERATING UNITS (if any) Department of Pathology, University of Maryland, School of Medicine, Baltimore, MD (E. M. McDowell, K. P. Keenan)		
LAB/BRANCH Laboratory of Experimental Pathology		
SECTION Respiratory Carcinogenesis Section		
INSTITUTE AND LOCATION NCI, NIH, Frederick, Maryland 21701-1013		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.1	1.2	1.9
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The induction of cancers from the epithelia of the different segments of the respiratory tract in animal models by multifactorial mechanisms is studied by treatments with combinations of chemical, physical and biological factors. Age at beginning of treatment is an important factor in the induction of respiratory tumors by diethylnitrosamine in a segment-specific manner. In hamsters treated from birth, there is an increased incidence and decreased latency of nasal cancers, in comparison with hamsters started at 8 weeks of age; no significant age-related effect was found on the induction of tracheal or lung neoplasms. Concurrent intraperitoneal injection of dimethylsulfoxide increased the incidence and severity and decreased the latency of respiratory tumors induced by intra-tracheal administration of suspensions of benzo[a]pyrene (BP) combined with ferric oxide. A complex multifactorial experiment of respiratory carcinogenesis in hamsters has been completed and the results are under analysis; preliminary results show that a single dose of N-methyl-N-nitrosourea (MNU) 2 weeks prior to a series of BP/ferric oxide administrations markedly enhances laryngeal and bronchial carcinogenesis relative to exposure to either carcinogen alone. Localized injury to the trachea enhances the carcinogenic effect of BP/ferric oxide not only in the trachea, but also in the bronchi. Previous exposure to BP/ferric oxide increases DNA binding of BP in all exposed segments of the respiratory tract, with the trachea showing the highest and the most transient binding levels.</p> <p>Silica-induced pulmonary epithelial proliferative lesions are studied for their pathogenetic relationship to granulomatous cell reaction and to cellular mediators of inflammation, in conjunction with long-term carcinogenesis studies of different forms of silica (quartz, cristobalite, tridymite).</p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

U. Saffiotti	Chief	LEP	NCI
S. F. Stinson	Biologist	LEP	NCI

Objectives:

The objective of this project is the elucidation of the mechanisms by which respiratory tract cancers, representing major forms of human cancer, are induced by chemical and physical factors, alone or in various combinations. Specific objectives are: (a) to characterize animal models for the induction of respiratory carcinogenesis, especially in relation to their human counterpart; (b) to investigate relevant host factors such as species, age and segmental responses in the respiratory tract (nasal mucosa, larynx, trachea, bronchi and peripheral airways); (c) to study the role of combined exposures to carcinogens and cofactors; (d) to study the mechanisms involved in respiratory carcinogenesis by carcinogens adsorbed on particulate materials and the role of particulates having toxic, fibrogenic and carcinogenic effects; (e) to analyze the metabolic pathways and DNA-binding of carcinogens in different segments of the respiratory tract and the role of cofactors; (f) to correlate in vivo effects with corresponding in vitro studies or cultured target tissues and cells.

Methods Employed:

Breeding of two specific pathogen-free colonies of Syrian golden hamsters, inbred 15:16/EHS:CR and outbred Syrian/CR:RGH. Establishment of lifetime and serially sacrificed colony control and treatment groups with general histopathological study and special investigation of respiratory tract cell differentiation and carcinogenesis. Rats of the F-344 strain and mice of several strains are also studied.

Intratracheal instillations of solutions and of particulate suspensions with characterization of particle distribution and retention. Study of respiratory tissue reactions by histologic, histochemical, autoradiographic and immunochemical methods and by scanning and transmission electron microscopy. Epithelial tissue isolation, fractionation and use for biochemical analysis of carcinogen localization, metabolism, and binding.

Carcinogens currently studied include polycyclic aromatic hydrocarbons and N-nitroso compounds; particulate materials currently include ferric oxide and silica (quartz, cristobalite, tridymite).

The effects of concurrent exposures to 3-methylindole (3MI), a cigarette smoke component, the solvent, dimethylsulfoxide (DMSO), and polycyclic aromatic hydrocarbons are being investigated in the hamster respiratory carcinogenesis model.

Major Findings:

(1) Differential age susceptibility to respiratory carcinogens. Diethylnitrosamine (DEN) was given subcutaneously for 12 weeks to outbred hamsters starting at 1 day, 4 and 8 weeks of age, for histogenesis and long-term carcinogenesis studies. Tumors of the nasal mucosa had an earlier onset, reached a much higher incidence and were more anaplastic in animals receiving DEN beginning at 1 day compared to those started at 8 weeks. The incidence of papillomas, which were localized primarily in the trachea and larynx and occasionally in the bronchi, was the same in either group, but multiplicity was greater in hamsters treated beginning at 8 weeks. Incidence, multiplicity or time of onset of bronchiolar alveolar tumors of Clara cell origin showed no relationship to age at beginning of treatment.

(2) Combined effects of topical and systemic factors on respiratory carcinogenesis.

a) Combined studies on 3-methylindole (3MI) with BP/Fe₂O₃. A study is nearing completion combining BP/Fe₂O₃ treatment with intraperitoneal administration of 3-methylindole (3-MI), which is known to induce selective damage to Clara cells and alveolar type I cells. Administration of the vehicle, DMSO, with or without 3-MI, was associated with a higher incidence and earlier onset of BP/Fe₂O₃-induced carcinomas in the larynx, trachea and bronchi than expected from BP/Fe₂O₃ controls. Since this finding suggests that DMSO may act as a modifier of carcinogenesis in this system, additional experimental groups have been set up to investigate this effect, by administering DMSO intraperitoneally at the time of the BP/Fe₂O₃ treatment (as above) or after a 5-day interval.

b) Combined studies on physical factors (microtrauma, saline and/or particulate instillation) with BP and/or N-methyl-nitrosourea (MNU). Experiments designed to analyze the isolated and combined effects of MNU, BP, Fe₂O₃, microtrauma and saline have been concluded and are being evaluated. A marked enhancement of laryngeal and bronchial carcinogenesis was observed in groups initiated with a single i.t. dose of MNU 2 weeks prior to BP/Fe₂O₃ administration. Also, cannulation injury to the trachea was found to increase the incidence of tracheal as well as bronchial neoplasms. This project is conducted in collaboration with the Department of Pathology, University of Maryland, under intramural support contract #N01-CP-25605 (see below for contract report). MNU, alone, also induced distant carcinogenic effects (ductular carcinomas of the pancreas and adrenal cortical carcinomas).

(3) [³H] Benzo[a]pyrene binding in vivo to tissue macromolecules from the trachea, bronchi and lung. In vivo binding of BP to DNA was studied after a single dose of [³H]BP/Fe₂O₃ was given intratracheally to hamsters that were either previously untreated or were pretreated with 11 weekly doses of cold BP/Fe₂O₃. DNA was isolated from trachea, bronchi and lungs at various times after administration of the radiolabelled dose. Pretreated hamsters had higher binding levels than previously untreated hamsters, in all segments. BP-DNA adducts reached a higher level in the trachea than in the bronchi and were lowest in the lung. Autoradiographic studies are under way to identify the cellular and segmental localization of [³H]BP.

(4) Respiratory carcinogenesis with crystalline silica in rats, mice and hamsters. Close interval serial sacrifice studies through 1 year, as well as lifetime observations, have been under way in F344 rats, 15.16/EHS:CR hamsters, and three strains of mice (A/J Cr, Balb/c and athymic nude) to compare the effects of various forms of crystalline silica (quartz, tridymite, cristobalite) on pulmonary fibrogenesis and carcinogenesis in these different species. Animals in specific pathogen-free (SPF) conditions will be compared with conventional animals. Studies in mice investigate the pulmonary adenoma response to the granulomatous reaction, and the role of immunocompetent cells is compared in normal and athymic nude mice. The reticuloendothelial cell reaction in all species is evaluated by histochemical and immunochemical methods in relation to epithelial hyperplasia and proliferation peripheral airways.

Publications:

Saffiotti, U.: Carcinogenic activity of silica and possible pathogenic role of the fibrogenic cellular reaction. (In Italian) Med. Lavoro 76: 351-357, 1985.

Saffiotti, U.: Comparability of in vitro and in vivo systems for carcinogenesis evaluations in different species, tissues and cells. In Vouk, V., Butler, G. C., Hoel, D. G. and Peakall, D. B. (Eds.): Methods for Estimating Risks of Chemical Injuries: Human and Nonhuman Biota and Ecosystems. Sussex, John Wiley and Sons, 1985, pp. 235-245.

Saffiotti, U.: Connecting molecular and cellular models with the corresponding events in tissues and organs: in vitro/in vivo comparisons in respiratory tract carcinogenesis. In Proceedings of 15th Conference on Environmental Toxicology. AF AMRL-TR-002, Air Force Base, OH, 1985, pp. 202-216.

Saffiotti, U.: Human lung cancer and experimental pathogenetic models: An increasingly close connection. In McDowell, E. M. (Ed.): Lung Carcinomas. Edinburgh, Churchill Livingstone, (In Press).

Saffiotti, U.: The pathology induced by silica in relation to fibrogenesis and carcinogenesis. In Goldsmith, D. F., Winn, D. M. and Shy, C. M. (Eds.): Silica, Silicosis and Cancer. Philadelphia, Praeger Publishers, 1986, pp. 287-307.

Schuller, H. M., Stinson, S. F., Ward, J. M., McMahon, J. B., Singh, G. and Katyal, S. L.: Loss of Clara cell antigens from neoplastic Clara cell derived lesions induced in the hamster lung by N-nitrosodiethylamine. Am. J. Pathol. (In Press).

Patents:

None

CONTRACT IN SUPPORT OF THIS PROJECT

UNIVERSITY OF MARYLAND (N01-CP-25605)

Title: Hamster Respiratory Carcinogenesis Resource for In Vivo/In Vitro
Correlation Studies

Current Annual Level: (Extended at no cost)

Man Years: 6.9

Objectives: To conduct in vivo respiratory carcinogenesis studies in the Syrian golden hamster and to provide in vitro techniques for the culture of respiratory epithelium to complement the in vivo studies.

Major Contribution: A large, long-term carcinogenesis experiment with the carcinogens, N-methyl-N-nitrosourea (MNU) and benzo[a]pyrene (BP), using 14 groups of Syrian golden hamsters, strain CR.RGH, was conducted to examine the effects of combinations of the following variables: single intralaryngeal instillation of MNU at 5 weeks of age, followed (after a 2-week interval) by a course of 15 canulations (once weekly), either at the larynx or in the trachea, with or without the instillation of saline, Fe₂O₃ in saline or BP/Fe₂O₃ in saline. All of the animals were examined histologically at the time of spontaneous death, at sacrifice when moribund, or at scheduled sacrifices at 25 or 78 weeks of age. Final pathology and statistical results are under evaluation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05276-05 LEP
PERIOD COVERED October 1, 1986 through September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Growth Control in Epithelial Cells and its Alteration in Carcinogenesis		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	M. E. Kaighn	Expert LEP NCI
Others:	D. G. Thomassen U. Saffiotti	Senior Staff Fellow LEP NCI Chief LEP NCI
COOPERATING UNITS (if any) Radiochemistry Division, Commission of the European Communities Joint Research Center, Ispra (Varese), Italy (F. Bertolero).		
LAB/BRANCH Laboratory of Experimental Pathology		
SECTION Tissue Culture Section		
INSTITUTE AND LOCATION NCI, NIH, Frederick, Maryland 21701-1013		
TOTAL MAN-YEARS: 1.9	PROFESSIONAL: 0.8	OTHER: 1.1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>An improved serum-free medium (LEP/MK2) has been developed for mouse keratinocytes (MK) and found to support continuous growth for at least 75 subcultures (more than 200 doublings). The established line has a reduced requirement for the factors in LEP/MK2. In addition, the responses to serum components decrease with time in culture. At high passage levels, sensitivity to growth inhibition by serum and by TGF-B was found to decrease with time, but was not abolished. MK cells rapidly lose their normal karyotype. By passage 4, they are essentially tetraploid with random gains and losses of individual chromosomes. These rapid chromosomal alterations may form a basis for the observed altered response to exogenous factors. The MK system is to develop a reliable selective assay for epithelial transformation and to investigate mechanisms of transformation by chemical carcinogens and oncogenes.</p> <p>For rat tracheal epithelial (RTE) cells, an improved serum-free medium (LEP/RTE 2) was developed, which supports clonal growth of primary RTE cells. The growth promoting activity of bovine pituitary extract, a critical component of LEP/RTE 2 medium, was found to be acid sensitive, heat stable, trypsin insensitive and had a molecular weight >5000. When the induction of preneoplastic RTE variants by the carcinogens, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and benzo[a]pyrene-diol-epoxide (BPDE), in cells plated in serum-containing medium on feeder cells or in serum-free medium was compared, MNNG was considerably more cytotoxic and potent as a transforming agent in serum-free medium than in serum-containing medium. The cytotoxic and transforming activities of BPDE were equivalent under both conditions. Development of serum-free media for both of these systems simplifies studies of altered growth control in carcinogenesis.</p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliation of Professional Personnel Engaged on this Project:

M. E. Kaighn	Expert	LEP	NCI
D. G. Thomassen	Senior Staff Fellow	LEP	NCI
U. Saffiotti	Chief	LEP	NCI

Objectives:

The overall objectives are to develop and characterize epidermal cell culture systems suitable for investigating chemical carcinogenesis and the sequence of changes produced by carcinogens in the control of growth and differentiation of epithelial cells. Mouse epidermal keratinocytes (MK) and rat tracheal epithelial cells (RTE) are used in these studies. Specific objectives include: (a) development of serum-free media for both MK and RTE cells and replacement of any undefined components with known factors, (b) development of selective media for cells transformed by oncogenes or chemical carcinogens, and (c) investigation of altered growth properties of normal cells with continued growth in culture.

Methods Employed:

BALB/c newborn mouse epidermal cells and F344 rat tracheal epithelial cells are isolated as previously described and cultured in serum-free media developed in this laboratory.

Clonal growth is measured by determining the colony-forming efficiency (CFE) or the average colony size after 7-10 days. The clonal growth rate is defined as population doublings/day (PD/d).

For transformation experiments, toxicity is determined by clonal survival assays. The appearance of altered growth properties, ultrastructure (using both SEM and TEM) and karyological changes are studied by standard methods.

Growth in soft agar and tumorigenicity in nude mice are used to demonstrate acquired neoplastic properties.

Major Findings:

Development and characterization of continuous mouse keratinocyte lines (MK) in serum-free medium. Since serum factor studies (Bertolero, et al., in press) showed that bovine serum albumin (BSA) stimulated the growth of 2⁰ keratinocytes, BSA (100 ug/ml) was added to original serum-free medium (LEP-1); the new formulation was called LEP/MK2. This new medium supported continuous passage of MK cells. One MK line has now been passed 75 times for more than 200 population doublings in LEP/MK2. Experiments to test the response of this established line to the components of the medium showed that later passage cells had simplified growth requirements. At passage 47, the line no longer had an absolute requirement for BPE, although deletion of this factor reduced growth by 60%. Modest

reductions in growth were observed when insulin (INS) or epidermal growth factor (EGF) were deleted. However, deletion of BSA or some other factors had little effect. A decreased sensitivity of later passage cells to serum, which is highly inhibitory at passage 2, was also found. A similar pattern of change was found with TGF-B. Inhibition of colony formation of MK cells by pure TGF-B leveled off at 45% (30 pg/ml). The established line also responds to high Ca^{+2} in the same way as 2^0 MK cells respond. As previously shown with 2^0 MK cells, the established line undergoes terminal differentiation with the same characteristic shift in morphology.

The chromosomal profiles of MK cells cultured in LEP/MK2 at passages 6, 17, and 34 have been studied by Giemsa banding. At passage 6, 88% were in the tetraploid range (75-85), 8% were in the diploid range (39-42) and the remainder (4%) had 150+ chromosomes. By passage 34, 100% of metaphases were in the tetraploid range. Two karyotypes were prepared from metaphases with 74 and 75 chromosomes. As with MK passage 6, there is, most probably, random chromosome losses and gains from metaphase to metaphase. No evidence for stable marker chromosomes was found in the metaphases studied. Passage 34 differs from passage 6 in that the small population of near diploid cells found in passage 6 is not present. The MK line is essentially tetraploid. Although chromosome studies on every early passage (2-4) are much more difficult, preliminary evidence indicates that the normal diploid karyotype is lost within 2-3 passages. It appears that the rapid loss of diploidy is not the result of growth in serum, but rather is an intrinsic characteristic of the mouse cells in vitro.

Development of serum-free medium for rat tracheal epithelial cells. RTE cells were previously grown in serum-containing medium on irradiated feeder cells (Thomassen, et al., *Cancer Res.* 43: 5956-5963, 1983). To eliminate the undefined roles of serum and feeder cells, a serum-free medium (LEP/RTE 1) was developed. This medium consists of Ham's F12 with 0.8 mM Ca^{+2} and 15 mM HEPES supplemented with bovine pituitary extract (1% v/v, BPE), bovine serum albumin (0.5 mg/ml, BSA), cholera toxin (1 nM, CT), epidermal growth factor (5 ng/ml, EGF), ethanolamine (50 uM, EA), hydrocortisone (0.3 uM, HC), insulin (5 ug/ml, INS), phosphoethanolamine (50 uM, PEA) and transferrin (5 ug/ml, TF). Although no single factor was found to be essential for the clonal proliferation of primary RTE cells, deletion of individual factors decreased either the number or size of the colonies. Multiple deletions revealed interactions between factors. Analysis of the growth promoting activity in BPE showed this activity to be acid sensitive, heat stable, trypsin insensitive and to have a molecular weight in the 3500-5000 range.

Transformation studies with RTE cells. In previous studies on carcinogen-induced transformation of RTE cells, serum was present in the medium conditioned by 3T3 feeder cells. The transformability of RTE cells in serum-free versus serum-containing medium was compared. Since serum inhibited the growth and stimulated differentiation of normal RTE cells, whereas enhanced growth (EG) variants were resistant to this effect, the use of serum as a selective agent was tested. Induction of EG variants by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and benzo[a]pyrene-diol-epoxide (BPDE) in RTE cells plated in serum-free medium or in serum-containing medium on feeder cells was compared. For each carcinogen, the maximum frequencies of induced variants were similar under the two culture

conditions. MNNG was more toxic and had a higher transforming activity in serum-free medium. In contrast, BPDE was equally cytotoxic and transforming under both sets of conditions.

Publications:

Bertolero, F., Kaighn, M. E., Camalier, R. F. and Saffiotti, U.: Effects of serum and serum-derived factors on growth and differentiation of mouse keratinocytes. In Vitro Cell. Dev. Biology. (In Press)

Kaighn, M. E.: Standards and testing procedures for media supplements, and supplies. In Vitro Monograph 5: 83-90, 1985.

Thomassen, D. G.: Role of spontaneous transformation in carcinogenesis: Development of preneoplastic rat tracheal epithelial cells at a constant rate. Cancer Res. 46: 2344-2348, 1986.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05381-03 LEP
PERIOD COVERED October 1, 1985 through June 20, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Cellular/Molecular Stages of Carcinogenesis in Respiratory Epithelia		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	D. Thomassen	Sr. Staff Fellow LEP NCI
Others:	U. Saffiotti	Chief LEP NCI
	M. E. Kaighn	Expert LEP NCI
COOPERATING UNITS (if any) Laboratory Oral Medicine, National Institute of Dental Research, NIH (M.I. Lerman)		
LAB/BRANCH Laboratory of Experimental Pathology		
SECTION Tissue Culture Section		
INSTITUTE AND LOCATION NCI, NIH, Frederick, Maryland 21701-1013		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.4	0.9	0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> The objective of this project is to identify and characterize changes at the cellular and molecular levels responsible for the development of preneoplastic and neoplastic variants of rat tracheal epithelial (RTE) cells. To identify cells having critical preneoplastic changes, comparative transformation experiments are described in which independent populations of preneoplastic RTE cells are treated with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or with cloned oncogene-containing DNAs from Harvey murine sarcoma virus (HaMSV, <u>ras</u>), polyoma virus, and MC29 virus (<u>myc</u>). Differential responses of the various cell lines to these treatments reveal differences in their preneoplastic potential. Using the hypothesis that differential responses of "preneoplastic" cell lines to carcinogens or oncogenic DNAs have a genetic basis, efforts will be directed towards the molecular cloning of the genes responsible for the conversion of preneoplastic to neoplastic cells. These studies require the development of assays suitable for the detection of these genes. The assay, based on the use of preneoplastic RTE cells for identification of RTE cell tumor genes, provides an intraspecific system in which the genes will be identified in cells that are genetically related to the cells in which the gene was originally active. Transfections of normal RTE cells with cloned oncogene-containing DNAs (<u>ras</u>, polyoma) induced escape from senescence but induced no complete neoplastic transformation. The latter was induced by transfection of oncogenes in colonies previously altered by treatment with MNNG (in all cell lines with polyoma and in some lines only with <u>ras</u>), indicating a multistep process of transformation. </p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

D. G. Thomassen	Senior Staff Fellow	LEP	NCI
U. Saffiotti	Chief	LEP	NCI
M. E. Kaighn	Expert	LEP	NCI

Objectives:

The overall goal of this project is to provide evidence on multistage carcinogenesis by identifying critical preneoplastic intermediates using the rat tracheal epithelial (RTE) cell system. Through the use of DNA transfection of cloned oncogenes, the role(s) and relationship(s) of exogenous oncogenes and the endogenous cellular homologues of oncogenes to RTE cell proliferation, differentiation, and transformation are studied.

Specific short-term objectives are: (1) comparison of preneoplastic rat tracheal epithelial (RTE) cell lines, which differ in their ability to progress to neoplasia, for the inducibility of neoplastic potential by treatment with the carcinogen, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), or by transfection with oncogenic DNAs; (2) to develop an assay using preneoplastic RTE cells to detect genes transferring the tumorigenicity phenotype; (3) to transfer the tumorigenicity phenotype by transfection using DNA from RTE tumor cells; (4) to begin cloning a gene from an RTE tumor cell which encodes the tumorigenicity phenotype; and (5) to transfect normal RTE cells with oncogenic DNAs and to compare the phenotypes obtained with those found after transformation of RTE cells with chemical carcinogens.

Methods Employed:

Neoplastic RTE cells are identified using the athymic nude mouse tumor assay. Athymic NCr nude mice are injected subcutaneously with cell suspensions and monitored for tumor formation. Treatment of RTE cells with MNNG is by a 4-hour treatment in HEPES buffered F12 medium without serum. DNA transfections using cloned oncogenes or high molecular weight DNAs are done using the calcium phosphate DNA precipitation technique. Transfection of normal RTE cells is done using culture conditions described in LEP Project #Z01CP05276-05. Clones of oncogene-transfected RTE cells are isolated following cotransfection of oncogene and pSV2-neo DNA and selecting for antibiotic G418-resistant clones. Carcinogen- or DNA-treated cells are assayed in nude mice for the induction of neoplastic potential. Molecular analyses of oncogene-transfected RTE cells include DNA and RNA isolation, RNA dot blotting, enzyme restriction of DNA, separation of DNA on agarose gels, Southern blotting, and Western blotting for some oncogene-encoded proteins. Techniques used for molecular cloning also include separation of DNA fragments on sucrose gradients and construction and screening of partial genomic libraries.

Major Findings:

Transfection of four preneoplastic RTE cell lines, enhanced growth (EG) variants, with pSV2-neo, pSV2-neo/ras or pSV2-neo/polyoma DNAs gave variable results depending on the EG variant and the oncogene(s) involved. Treatment of the EG variants with pSV2-neo did not change their tumorigenic potential. Two of four EG variants were nontumorigenic after pSV2-neo treatment, while the other two variants were partially tumorigenic. All four EG variants were highly tumorigenic after treatment with pSV2-neo/polyoma, demonstrating that oncogene-containing DNA can be taken up and expressed by these cells. Treatment with pSV2-neo DNA increased the tumorigenicity of one partially tumorigenic EG variant and one nontumorigenic EG variant, while the tumorigenicity of the other two variants was not affected.

Oncogene-containing plasmids had a marked effect on cell proliferation and morphology. Colonies which formed from cells treated with pSV2-neo/ras and pSV2-neo/polyoma were two to three times larger than those which formed from cells treated with only pSV2-neo, due to increased cell proliferation. In addition, the cells in pSV2-neo/oncogene-induced colonies were often less flattened and more spindle shaped than cells in pSV2-neo-induced colonies.

EG variants transfected with pSV2-neo/ras were examined for the presence of transfected ras DNA, expression of ras-specific RNA and expression of ras-specific p21 protein. Although the majority of EG variants transfected with pSV2-neo/ras contained transfected ras DNA, there was not a correlation between the presence of transfected ras DNA, elevated levels of ras-specific RNA, elevated levels of p21 expression and tumorigenicity.

Normal RTE cells in serum-free culture could serve as recipients for exogenous DNA in transfection experiments. As above, oncogene-containing plasmids had a marked effect on RTE cell proliferation and morphology. Colonies formed after pSV2-neo/oncogene treatment were larger than those formed after pSV2-neo treatment alone. Cells treated with pSV2-neo or pSV2-neo/ras were morphologically the same as untreated RTE cells. Cells treated with pSV2-neo/polyoma were initially the same as untreated RTE cells; however, with time (2 to 4 wks after treatment), focal areas of morphologically altered cells appeared within RTE cell colonies which were highly refractile, tightly packed and often piled-up. In addition, pSV2-neo/polyoma-induced colonies continued to grow in serum containing medium, whereas pSV2-neo and pSV2-neo/ras-induced colonies underwent squamous differentiation in the same medium. Cells from pSV2-neo/polyoma-induced colonies could be subcultured and passaged as cell lines in either serum-free or serum containing medium. Some of these cells have been injected into nude mice and no tumors formed after more than 3 months in contrast to pSV2-neo/polyoma transfected EG variants which were nearly always tumorigenic within 3-4 wks.

Publications

None

Patents

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05494-01 LEP
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Cellular and Molecular Studies in Normal and Neoplastic Human Prostatic Cells		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	M. E. Kaighn	Expert LEP NCI
Others:	D. G. Thomassen J. F. Lechner	Sr. Staff Fellow LEP NCI Microbiologist LHC NCI
COOPERATING UNITS (if any) Departments of Urology and Surgery, Northwestern University Medical School (J. Kozlowski); Laboratory of Oral Medicine, National Institute of Dental Research (M. I. Lerman); University of Texas Medical School (D. Sirbasku)		
LAB/BRANCH Laboratory of Experimental Pathology		
SECTION Tissue Culture Section		
INSTITUTE AND LOCATION NCI, NIH, Frederick, Maryland 21701-1013		
TOTAL MAN-YEARS: 0.8	PROFESSIONAL: 0.2	OTHER: 0.6
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) A major goal of this project is to detect and clone a transforming gene from the human prostatic cancer cell line, PC-3. Transforming activity has been found in PC-3 genomic DNA by the NIH/3T3 cell focus assay. Using the nude mouse tumorigenicity assay in conjunction with drug selection of NIH/3T3 cotransfected cells, the presence of an activated oncogene was confirmed. This unidentified oncogene, which does not appear to be a member of the <u>ras</u> family, is being cloned by sib selection using human repeated sequences as a probe. Preliminary results suggest the possible presence of suppressor DNA sequences that inhibit tumorigenicity of certain NIH/3T3 cells. Isolation of this suppressor activity also has been undertaken by sib selection. Autocrine activities of PC-3 and its highly metastatic variants are being investigated in collaboration with D. Sirbasku (University of Texas). An ascites line (PC-3asc) was isolated directly in serum-free medium from a tumor obtained by inoculation of PC-3/mA2 in the nude mouse (in collaboration with J. Kozlowski). The PS-3asc line was found to produce an autogenous factor that stimulated its own growth at low cellular inocula. In addition, preliminary data indicate that medium conditioned by PC-3asc cells has TGF activity in normal rat kidney (NRK) cells in soft agarose. This putative TGF is being isolated and will be further purified by standard biochemical procedures. Karyotypic analysis has shown that the PC-3asc line has many features in common with the parental line, PC-3/mA2, except for a few marker chromosomes with homogeneously staining region and numerous minute and double minute chromosomes.		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

M. E. Kaighn	Expert	LEP	NCI
D. G. Thomassen	Sr. Staff Fellow	LEP	NCI
J. F. Lechner	Microbiologist	LHC	NCI

Objectives:

The cellular and underlying molecular changes in normal prostatic epithelium that lead to metastatic prostatic cancer are being studied in well-characterized normal and neoplastic cell lines. Specific objectives include: (a) transformation of normal human prostatic epithelial cells by transfection of known oncogenes and genomic DNA from prostatic cancer cells, (b) analysis of gene activation and transforming growth factor activity in metastatic variants of the prostatic carcinoma line, PC-3, and (c) isolation and cloning of a transforming gene from PC-3.

Methods Employed:

A normal human prostatic epithelial cell line (NP-2s) and a prostatic adenocarcinoma line (PC-3) are available from previously established frozen stocks (Lechner, J. F., et al., JNCI 60: 797-801, 1978; Kaighn, M. E., et al., Invest. Urol. 17: 16-23, 1979). Media and culture methods are as previously reported. Transfection of oncogenes and genomic DNA is by the calcium phosphate procedure. Transforming activity of DNAs assessed in the NIH/3T3 cell focus assay or by injection into nude, athymic mice. The procedure of Brown, R., et al. (Carcinogenesis 5: 1323-1328, 1984) is used as described with Ecogpt selection (Mulligan, R. C. and Berg, P., Proc. Natl. Acad. Sci. USA 78: 2072-2076, 1981).

Purification of cellular, phage and plasmid DNAs is according to standard procedures, as is purification of total, nuclear, and poly A⁺ RNAs. Genes are isolated from libraries through the use of the sib selection protocol, since they are present in biologically active form. The original library is divided into a number of pools, each of which is amplified and assayed for the desired gene activity. With each successive round, the number of recombinants in positive pools is reduced by the number of pools. Finally, a pool containing a few independent recombinants is isolated and each recombinant is tested individually. The same protocol is applied to transfected cells, which represent "cell libraries" and are assayed for tumorigenicity in nude mice.

Standard recombinant DNA procedures and enzymatic reactions to construct phage and plasmid libraries, subcloning of desired sequences into suitable vectors and screening libraries with specific probes are employed. The structure of isolated cloned genes will be established by DNA sequencing, restriction and genomic mapping, and computer-aided analyses (Maniatis, T., Fritsch, E. F. and Sambrook, J. (Eds.): Molecular Cloning. Cold Spring Harbor, Cold Spring Harbor Laboratory, 1982, 545 pp.; Wilbur, J. and Lipman, D., Proc. Natl. Acad. Sci. USA 80: 726-731, 1983).

Autocrine or transforming growth factors (TGFs) are isolated from serum-free medium conditioned by the growth of cells. These factors are further purified by established biochemical methods and assayed for TGF activity in soft agarose using NRK cells as the indicator cell line.

Major Findings:

Work on this project has led to (1) the isolation of a new variant of PC-3 metastatic to the ascitic fluid in nude mice (PC-3asc), (2) identification of a transforming gene for NIH/3T3 cells in PC-3, and (3) tentative identification of a human gene that suppresses the spontaneous tumorigenicity of NIH/3T3 cells in nude mice.

The metastatic capability of the prostatic adenocarcinoma line, PC-3, in nude mice is being investigated in collaboration with Dr. James M. Kozlowski. The incidence of metastasis was increased by the isolation of variant sublines from secondary tumor deposits and by the use of an advantageous inoculation site (Kozlowski, J. M., et al., *Cancer Res.* 44: 3522-3529, 1984). The basis of this enhancement of metastatic phenotype may be correlated with different responses to growth factors, substrates and activated or amplified oncogenes.

Experience had indicated that the presence of serum in the culture medium, even transiently, suppressed the synthesis of TGFs (Sirbasku, D. personal communication). For this reason, we isolated PC-3asc directly in serum-free medium from a nude mouse tumor. This new line (PC-3asc) was found to have the isozyme pattern and karyotype of PC-3. Twelve marker chromosomes were identified in the PC-3asc line. Eleven of these were also found in the PC-3/mA2 line. The PC-3asc line lacks the marker chromosome, M7, and has a new marker chromosome, M13. That marker chromosome has a homogeneously staining region (HSR), which is associated with gene amplification. Amplification is also indicated in that some marker chromosomes are duplicated to a greater extent than was observed in the parent line. Another difference between PC-3asc and PC-3/mA2 that was observed in 10 to 20 percent of the population was the presence of minute and double minute chromosomes. Double minutes, as well as HSRs, are known to be indicators of gene amplification.

In serum-free medium, the growth of PC-3 is population-dependent, indicating the secretion of an autocrine growth factor. Pooled serum-free conditioned medium (CM) from PC-3asc was dialyzed in Spectrapore dialysis tubing (3500) vs 1% acetic acid in the cold, clarified by centrifugation and lyophilized. A preliminary assay showed that this crude concentrate stimulated the growth of NRK colonies in soft agarose. Further purification of the acidic concentration on Bio-Gel P-60 is in progress.

To determine whether human prostatic carcinoma lines contained transforming activity, high-molecular-weight DNA was extracted from PC-3/mA2 cells using gpt selection to decrease the probability of including spontaneous transformants. NIH/3T3 cells were cotransfected with PC-3/mA2 and pSV2-gpt as described in Methods. Calf thymus DNA and calf thymus DNA plus pSV2-gpt served as controls. Two days after treatment the cells were subcultured into gpt selective medium.

Large colonies developed by 2 weeks. No colonies developed in dishes without the gpt gene. Twenty-seven dishes, containing an estimated 3753 colonies from the cotransfected group (C), were divided into nine pools of three dishes each. Thus, each pool consisted of 400 independent colonies. The nine pools were separately expanded to provide sufficient cells for determination of tumorigenicity in nude mice.

Cells from selected tumors that appeared at week 5 were grown into mass cultures in selective and nonselective media and their DNAs were analyzed by Southern blotting for the presence of human repetitive sequences by using the BLUR-8 plasmid probe. All tested cell lines from these primary tumors contained high levels of human Alu repeat sequences. DNAs from the same lines did not contain the three ras oncogenes, N-, Ha- or Ki-ras by Southern blotting.

The ability of DNAs from primary tumors to induce rounds of secondary tumors after transfection into NIH/3T3 cells has been confirmed. Surprisingly, in the assays reported above, several pools remained nontumorigenic for 6 weeks when controls showed a high incidence of tumor formation. One pool did not induce tumors even after 8 weeks, at which time all control mice injected with calf thymus DNA-transfected NIH/3T3 cells had developed tumors. This pool may contain sequences that suppress spontaneous tumors. Identification of a putative suppressor gene is under way by the sib selection protocol applied to transfected cells assayed in nude mice for inhibition of tumorigenicity.

Publications:

None

Patents:

None

ANNUAL REPORT OF
THE LABORATORY OF HUMAN CARCINOGENESIS
CHEMICAL AND PHYSICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1985 through September 30, 1986

The Laboratory of Human Carcinogenesis conducts investigations to assess (1) mechanisms of carcinogenesis in epithelial cells from humans and experimental animals, (2) experimental approaches in biological systems for the extrapolation of carcinogenesis data and mechanisms from experimental animals to the human situation, and (3) host factors that determine differences in carcinogenic susceptibility among individuals.

The scientific and managerial strategy of the Laboratory is reflected in its organization into three sections, i.e., In Vitro Carcinogenesis Section (IVCS), Carcinogen Macromolecular Interaction Section (CMIS), and Biochemical Epidemiology Section (BES). Scientifically, the emphasis is on the role of inherited or acquired host factors as important determinants in an individual's susceptibility to carcinogens and cocarcinogens. Our investigations of host factors involve interspecies studies among experimental animals and humans, cover the spectrum of biological organization ranging from molecular and cellular biology, pathology, epidemiology, and clinical investigations. Two sections (IVCS and CMIS) devote their major efforts to more fundamental and mechanistic studies. The scientific findings, techniques, and concepts developed by these two sections and, of course, the scientific community at large, are utilized by the BES in selected and more applied studies of carcinogenesis and cancer prevention. The laboratory-epidemiology studies in this section require the expertise found in the IVCS and CMIS and in the NCI Epidemiology Program. The Laboratory requires unique and complex resources. For example, collection of viable normal and neoplastic epithelial tissues and cells--well characterized by morphological and biochemical methods from donors with an epidemiological profile--requires continued cooperation among donors and their families, primary care physicians (internists, surgeons, house staff), surgical pathologists, nurses, epidemiologists, and laboratory scientists.

Since its establishment, LHC has been fortunate to have the constructive criticism of a group of colleagues who are recognized experts in molecular biology (Carlo Croce, M.D., Associate Director, Wistar Institute, Philadelphia, PA); cell biology (Ted Puck, Ph.D., Director, Eleanor Roosevelt Center for Cancer Research, Denver, CO; David Prescott, Ph.D., Distinguished Professor, Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, CO), and biochemistry (Allan Conney, Ph.D., Director, Department of Biochemistry, Roche Institute of Molecular Biology, Nutley, NJ). These colleagues visit LHC on an individual basis at least once a year and review ongoing research projects with LHC staff. The exchange of ideas and their continuing advice have made these visits invaluable.

In addition to the frequent and informal exchanges of information among LHC staff, the Laboratory and each section have monthly scientific and administrative meetings. We also sponsor, with the Laboratory of Cellular Carcinogenesis and Tumor Promotion (LCCTP), a weekly Journal Club. A monthly joint rotating seminar series is presented by LHC, LCCTP, and the Laboratory of Experimental Carcinogenesis. LHC sponsors seminars with extramural speakers at monthly intervals.

LHC also organizes meetings of the Human Studies Collaborative Group, which take place in Bethesda and include participants from the NIH scientific community, extramural experts and collaborators, and staff from LHC resource contracts. These biannual meetings provide a forum for the survey of an ongoing research area, e.g., respiratory carcinogenesis, for informal discussions.

RESEARCH STRATEGY

A central problem in carcinogenesis research is the extrapolation of data and knowledge of mechanism from experimental animals to the human population, and within this heterogeneous population, extrapolation among individuals. A subset of this problem is the difficulty associated with extrapolation from one level of biological organization to another, i.e., molecules to macromolecules to organelles to cells to tissues to organs to intact organisms.

The strategy currently employed by LHC is a refinement of the one we formulated a decade ago. Epidemiologic and clinical observations provide clues as to how we should conduct our investigations. In many cases, clinical investigations and studies using animal models can be used to test hypotheses. In other cases, in vitro models are more suitable. These models utilize human tissues and cells collected at the time of immediate autopsy (i.e., from organ donors) and surgery. Remarkable progress has been made during the last several years in the development of methodology to culture normal human tissues and their epithelial cells from most major sites of human cancer. Therefore, the mechanisms of action of carcinogens, tumor promoters, growth factors, differentiation inducers, etc., can be investigated at the tissue, cellular, and subcellular levels of biological organization. An integral facet of this strategy is that the same types of tissues and cells from experimental animals can be maintained in identical, controlled in vitro experimental settings so that comparative studies using human and experimental animal material can be conducted.

After developing in vitro models, we are systematically investigating several research areas, i.e., (I) In Vitro Model Development, (II) Carcinogen Metabolism, DNA Damage, and DNA Repair, (III) Cell and Molecular Biology of Normal and Neoplastic Cells, and (IV) Carcinogenesis and Related Studies (Table 1). In recent years, the majority of the research projects have progressively shifted from areas I and II to areas III-V. As noted above, knowledge acquired through these studies is then used in our investigations of Biochemical and Molecular Epidemiology of Human Cancer (V). Although we have developed in vitro models for several types of human tissues, the emphasis has been on lung, esophagus, and colon. Summaries of research projects in each of these integrated areas of our research program are listed in the following sections.

TABLE 1. STRATEGY FOR STUDYING HUMAN CARCINOGENESIS

- I. In Vitro Model Development
 - A. Collection of Human Tissues and Cells
 - B. Explant and Cell Cultures
 - C. Serum-Free Culture Conditions
- II. Carcinogen Metabolism, DNA Damage and DNA Repair
 - A. Interspecies Comparisons
 - B. Interindividual Comparisons
 - C. Intercellular Comparisons
 - D. Cell-Mediated Responses
 - E. Others
- III. Cell and Molecular Biology of Normal and Neoplastic Cells
 - A. Growth Factors
 - B. Inducers of Differentiation
 - C. Cytoskeleton
 - D. Cytokeratins
 - E. Others
- IV. Carcinogenesis and Related Studies
 - A. Oncogenes
 - B. Chemical Carcinogens
 - C. Physical Carcinogens
 - D. Oncogenic Viruses
 - E. Chemical-Microbial Interactions
 - F. Tumor Promoters, Aldehydes and Peroxides
 - G. Others
- V. Biochemical and Molecular Epidemiology
 - A. Laboratory-Clinical Investigations
 - B. Animal Models
 - C. Others

I. IN VITRO MODEL DEVELOPMENT

Remarkable progress has been made during the last few years by this and other laboratories in establishing conditions for culturing human epithelial tissues and cells. Normal tissues from most of the major human cancer sites can be successfully maintained in culture for periods of weeks to months. We have developed chemically defined media for long-term culture of human bronchus, colon, esophagus, and pancreatic duct. Primary cell cultures of human

epithelial outgrowths have been obtained from many different types of human tissues. Isolated epithelial cells from human bronchus and esophagus can be transferred three or more times and can undergo more than 30 cell divisions. Human bronchial and esophageal epithelial cells can also be grown in serum-free culture medium. Morphological, biochemical, and immunological cell markers have been used to identify these cells as unequivocally of epithelial origin.

Clonal growth of normal human pleural mesothelial cells in a serum-free, as well as serum supplemented culture medium, has also been achieved so that the in vitro transformation, by asbestos, of these cells can be studied.

The availability of nontumorous epithelial tissues and cells that can be maintained in a controlled experimental setting offers an opportunity for the study of many important problems in biomedical research, including carcinogenesis. For example, the response of human bronchial epithelial cells (enhanced growth or differentiation) after either (a) exposure to carcinogens and/or tumor promoters or (b) DNA transfection by oncogenes is being actively investigated. Parallel investigations using epithelial tissues and cells from experimental animals allow investigators to study interspecies differences in response to carcinogens, cocarcinogens, and anticarcinogens.

In Vitro Models for Lung Carcinogenesis Investigations

Defined culture conditions that permit normal adult human bronchial epithelial (NHBE) cells to multiply at a clonal growth rate of 0.65 population doublings per day have been developed. This has been accomplished by (a) optimizing the calcium concentration and osmolarity of nutrient medium, MCDB 151; (b) supplementing with purified factors (epidermal growth factor, 5 ng/ml; transferrin, 10 µg/ml; hydrocortisone, phosphoethanolamine, and ethanolamine, each at 0.5 mM; and trace elements); and (c) coating the surface of the culture dish with a mixture of fibronectin, collagen, and bovine serum albumin (FN/C/BSA). The clonal growth rate can be increased to more than one population doubling per day by supplementing the defined medium with bovine pituitary extract (BPE; 35 µg/ml), epinephrine (0.25 µM), triiodothyronine (10 nM), and retinoic acid (0.3 nM). Using either the defined system or the BPE-containing media, cultures of bronchial epithelial cells obtained from more than 150 non-cancerous donors have been subcultured at clonal densities with a colony forming efficiency of 2-4%. In addition, high density cultures have been subcultured more than five times with four to six population doublings per passage.

Serum supplementation of the growth media reduces the growth rate in a dose-dependent manner. This discovery explains why previously it had proven difficult to establish replicative cultures of NHBE cells, e.g., serum contains factors that cause cells to cease dividing. We have determined that the resultant effect of the growth-inhibiting action of serum is to cause the cells to undergo squamous differentiation, e.g., the cells increase in area, multilayer, acquire tonofilaments and desmosomes, and produce cross-linked envelopes. The potency of serum can be controlled by other medium constituents, e.g., Ca^{2+} and cholera toxin, which increase and neutralize the effectiveness of the serum dose, respectively.

Methods have also been developed to culture pleural mesothelial cells obtained from non-cancerous donors. Pure cultures are initiated by pelleting the mesothelial cells from pleural effusion fluid and inoculating the resuspended cells into FN/C/BSA-coated dishes containing LHC basal nutrient medium supplemented with growth factors. Using this protocol, mesothelial cell cultures have been established from more than 200 donors. The cells can be subcultured at clonal density with a colony-forming efficiency of more than 10% and high density cultures can be subcultured four to six times before senescence.

These cultures systems are now sufficiently established to permit 1) pathobiologic investigations of NHBE cells, e.g., aberrant growth control processes, differentiation, and carcinogenesis using conditions that isolate the results from the influence of serum and feeder cells, and 2) short- and long-term asbestos carcinogenesis investigations of mesothelial cells.

Studies of Proliferative Response in Normal Human Bronchial Epithelial Cells

We investigated the mitogenic effects of compounds on normal human bronchial epithelial (NHBE) cells cultured in a defined serum-free medium using clonal growth and colony-forming efficiency assays. We also measured ornithine decarboxylase activity and cyclic AMP levels, since polyamine and cAMP metabolism are involved in cell proliferation in most systems. Many compounds were investigated, including human pituitary growth hormone, testosterone, estradiol, estriol, calcitonin, fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) and most had no mitogenic effect. Epidermal growth factor (EGF), bombesin, and the C-terminal tetradecapeptide of gastrin-releasing peptide (GRP₁₄₋₂₇), and human chorionic gonadotropin (HCG) each independently increased the clonal growth rate significantly. An aqueous extract of bovine pituitaries (BPE) had no effect by itself but did increase the clonal growth rate when EGF was also present. Cyclic-AMP-enhancing agents were mitogenic only when both EGF and BPE were present.

II. CARCINOGEN METABOLISM, DNA DAMAGE AND DNA REPAIR

The earliest events in the multistage process of chemical carcinogenesis are thought to include (1) exposure to the carcinogen; (2) transport of the carcinogen to the target cell; (3) activation to its ultimate carcinogenic metabolite, if the agent is a procarcinogen; and (4) DNA damage leading to an inherited change. Therefore, one important use of cultured human tissues has been in the investigation of the metabolism of chemical carcinogens because many environmental carcinogens require metabolic activation to exert their oncogenic effects; the metabolic balance between carcinogen activation and deactivation may, in part, determine a person's oncogenic susceptibility; and knowledge of the comparative metabolism of chemical carcinogens among animal species will aid efforts to extrapolate data on carcinogenesis from experimental animals to humans. We and our coworkers have systematically examined the metabolism of procarcinogens of several chemical classes which are considered to be important in the etiology of human cancer. Procarcinogens of several chemical classes can be activated enzymatically to electrophilic reactants that bind covalently to DNA in cultured human tissues. The studies of activation and deactivation of representative procarcinogens have revealed that the metabolic pathways and the predominant adducts formed with DNA are generally similar between humans and experimental animals. Wide quantitative interindividual differences (50- to 150-fold) are found in humans and other

outbred animal species. When the metabolic capabilities of specimens from different levels of biological organization are compared, the profile of benzo[a]pyrene metabolites is similar in cultured tissues and cells, but subcellular fractions, e.g., microsomes, produce a qualitative and quantitative aberrant pattern.

Although DNA repair has been extensively studied in human fibroblasts, lymphoid cells, and neoplastic cells, little information is available concerning DNA repair in normal human epithelial cells. Using the methodology to culture human bronchial epithelial and fibroblastic cells developed in our laboratory, we have initiated studies to investigate DNA damage and repair caused by chemical and physical carcinogens as examined by alkaline elution methodology, BND cellulose chromatography, unscheduled DNA synthesis, and high pressure liquid chromatographic analysis of the formation and removal of carcinogen-DNA adducts. As we reported last year, human bronchial epithelial cells repair single-strand breaks in DNA damaged by X-radiation, UV-radiation, chromate, polynuclear aromatic hydrocarbons, formaldehyde, or N-nitrosamines at rates similar to bronchial fibroblasts.

Repair of Carcinogen-Induced Damage in Human Epithelial and Fibroblast Cells

Normal human bronchial epithelial cells (NHBE) were characterized with regard to DNA damage and repair resulting from environmental lung carcinogens. In most aspects, bronchial epithelial cells resembled bronchial fibroblasts. The O⁶-alkylguanine-DNA alkyltransferase repair system, as well as gamma, UV, and benzo(a)pyrene diol epoxide (BPDE) repair, were strikingly inhibited by the aldehyde class of lung carcinogens such as formaldehyde. Correspondingly, formaldehyde potentiated the mutagenicity of N-methyl-N'-nitrosourea. Formaldehyde also depleted cellular stores of protective thiols.

The Genotoxicity of Fecapentaene-12

Higher levels of mutagens in the feces of certain populations eating a western diet have been shown to correlate with an increased risk of colon cancer. Ninety percent of this mutagenicity can be accounted for by a group of compounds called fecapentaenes, which are potent direct-acting mutagens in the Ames *Salmonella* assay. Although fecapentaenes may play a role in the etiology of human colon carcinoma, their genotoxic effects have not been previously studied in human cells. We have established that fecapentaene-12 (fec-12), a prototype for this group of compounds, is cytotoxic, mutagenic, and causes DNA single strand breaks in cultured human fibroblasts. These results indicate that fec-12 is genotoxic in human cells and are consistent with the hypothesis that fecapentaenes may be involved in the pathogenesis of human colon cancer. Plasmid assays investigating the nature of fec-12 interactions with DNA have shown that this compound causes interstrand DNA cross-links. Results from electron microscopic studies support these findings and also indicate that fec-12 directly causes DNA single strand breaks.

III. CELL AND MOLECULAR BIOLOGY OF NORMAL AND NEOPLASTIC HUMAN CELLS

Our operational definitions of normal, premalignant, and malignant cells are biological (e.g., differentiated state, growth, altered cellular affinities and architecture) and tumorigenicity when injected into the appropriate host. Methods for the culture of human epithelial tissues and cells provide

an opportunity to investigate the biology and molecular mechanisms controlling growth and differentiation of normal and neoplastic cells.

Control of Growth and Differentiation of Human Bronchial Epithelial Cells and Their Malignant Counterparts

Defined methods to grow replicative cultures of normal human bronchial epithelial (NHBE) cells without serum have been developed. These cells can be subcultured several times and will undergo 35 population doublings. They have the anticipated epithelial cell characteristics of keratin, desmosomes, and blood group antigens on their cell surface. NHBE cells inoculated at clonal density will multiply with an average generation time of 28 hours. The majority of the cells are small and migratory and have few tonofilaments. Adding human or bovine whole blood-derived serum (BDS) depresses the clonal growth rate of NHBE cells in a dose-dependent fashion.

BDS reduces the clonal growth rate of NHBE cells by specifically inducing squamous differentiation. The differentiation-inducing activity was not present in plasma but was found in platelet lysates. Thus, purified platelet factors were assayed for differentiation-inducing activity. Platelet-derived growth factor proved to be inactive. However, NHBE cells were shown to have high affinity receptors for a second platelet constituent, transforming growth factor type β (TGF- β), and incubating NHBE cells with TGF- β induced the following markers of squamous differentiation: 1) increase in Ca ionophore-induced formation of cross-linked envelopes; 2) increase in extracellular activity of plasminogen activator; 3) irreversible inhibition of DNA synthesis; 4) decrease in clonal growth rate; and 5) increase in cell surface area.

The IgG fraction of anti-TGF- β antiserum prevented both the inhibition of DNA synthesis and the induction of differentiation by either TGF- β or BDS. Therefore, TGF- β is the primary differentiation-inducing factor in serum for NHBE cells.

Epinephrine-antagonized TGF- β induced both inhibition of DNA synthesis and squamous differentiation in NHBE cells. Although epinephrine increased cyclic AMP (cAMP) levels in NHBE cells, TGF- β did not alter the cAMP levels in NHBE either in the presence or absence of epinephrine. Therefore, the antagonistic effects of epinephrine on TGF- β induction of differentiation appear to occur via indirect mechanisms.

Patterns of Expression of Epithelial Differentiation Markers (Keratins, Involucrin, and Cross-Linked Envelopes) During the Course of Normal Differentiation and Development and in Neoplasia

We have characterized the pattern of expression of the main markers of epithelial differentiation; namely, keratins, involucrin, and cross-linked envelopes, in human epithelial derived from major cancer sites (in particular, skin, esophagus, and lung, as well as others) during the course of terminal

differentiation in embryonic development, postnatal maturation, and in neoplasias. The pattern of keratin expression was found to be dependent on the cell type, the stage of differentiation and/or development, and the extrinsic environment of cell. During in vitro cultivation, human keratinocytes retained many aspects of their distinctive programs of differentiation.

Neoplastic epithelial cells, while they continued to maintain keratin expression, both in vivo and in culture, frequently exhibited altered keratin patterns. Immunocytochemical and/or immunoprecipitation studies with keratin antiserum (against either total or individual keratins) revealed that the keratin pattern was dependent on the tumor type and the degree of tumor differentiation and was useful in the diagnosis and classification of many tumors, and in elucidating the histogenesis of certain tumors. In skin tumors (basal and squamous cell carcinoma), abnormal keratin profiles were observed in histologically normal-appearing or hyperplastic epidermis adjacent to tumor, indicating abnormal squamous maturation.

Examination of the pattern of expression of other markers of terminal keratinocyte differentiation (involucrin and cross-linked envelopes) in a wide variety of normal and neoplastic tissues revealed that involucrin and cross-linked envelopes were sensitive and specific markers of squamous and urothelial differentiation and of squamous- and urothelial-derived malignancies. Staining for involucrin was useful in distinguishing benign from malignant urothelial and squamous lesions. Benign squamous and urothelial lesions and grade I papillary urothelial carcinomas maintained a normal involucrin distribution pattern, whereas higher grade papillary urothelial carcinomas, infiltrating urothelial and squamous carcinomas, and in situ urothelial and squamous carcinomas demonstrated abnormal staining patterns with intense staining for involucrin frequently localized to individual larger tumor cells at all levels of the epithelium or in areas of squamous differentiation. The presence of involucrin in the tumors correlated with the degree of squamous differentiation of the tumor cells and in the case of skin neoplasms, the presence of large keratins. Foci of squamous differentiation in adenocarcinomas and other epithelial malignancies stained strongly for involucrin. Similar to results with keratin, abnormal patterns of involucrin staining (increased staining into the lower spinous layers of epidermis) were noted in nonneoplastic epidermis adjacent to squamous cell carcinomas and overlying basal cell carcinomas of the skin. This type of staining pattern was also prevalent in benign epidermal hyperplasia, indicating that this altered staining pattern was not specific for carcinomas. Staining for involucrin also provided evidence as to the derivation of certain tumors, specifically Brenner tumors of the ovary and Walthard rests of the fallopian tube, lesions of uncertain histogenesis. Demonstration of involucrin within the epithelium of these tumors supported their derivation from either coelomic epithelium by a process of urothelial metaplasia or possibly from urogenital rests.

Analysis of Pathways Involved in Terminal Differentiation of Human Bronchial Epithelial Cells

A cell culture system has been defined in which primary human bronchial epithelial cells are induced to differentiate by transforming growth factor

type beta (TGF- β) or 12-O-tetradecanoylphorbol-13-acetate. Initial analyses of control and differentiating cells by two-dimensional gel electrophoresis suggest that a limited number of proteins are modulated in this process.

Cell Surface Antigen Expression - Normal and Neoplastic Cells

Cell surface antigen expression was investigated in neoplastic cells, lymphoid and non-lymphoid, using monoclonal antibodies and the fluorescence-activated cell sorter. Acute lymphocytic leukemia cells were studied for their expression of cell surface markers which define functional subpopulations of human T-lymphocytes. The patterns of expression of antigens on these cells differed from the combination of antigens seen in the normal, mature non-malignant T-cells. The antibody 3A1, which detects an antigenic determinant on all normal T-cells, was expressed on T-cell acute lymphocytic leukemia cells and in relatively low levels on null cell leukemias.

Normal bronchial epithelial cells transformed with the Harvey ras oncogene were studied for cell surface antigens. The antigenic profile did not differ significantly from the normal bronchial epithelial cells; however, when these cells were passed into nude mice and the tumor was explanted and grown in culture, a decrease in the amount of HLA class I antigens and an increase in the expression of HLA class II antigens was observed.

Alterations in the expression of cell surface antigens which correlate with particular functional subsets of T lymphocytes are being studied in a normal population. This study was initiated after we observed alteration in the numbers of T-cell subsets in patients with AIDS and in the AIDS at-risk population. Lymphocytes from 600 disease-free individuals who consented to a limited questionnaire (inquiring into smoking status, occupation, and over-the-counter drug use) have been obtained and are being studied for expression of cell surface antigenic determinants. Preliminary results demonstrate an alteration in the ratio of T4⁺ to T8⁺ lymphocytes in smokers. Ratios also appeared to be different depending upon age and race of the individual.

Cytosine Methylation and Cellular Physiology and Pathology

The role DNA methylation plays in the promoter regions of two separate gene systems has been clarified. Demethylation of at least one Hpa II restriction site upstream from the structural coding sequences of human gamma-globulin or the hepatitis B core antigen gene is necessary but not sufficient for the initiation of transcription. The final conversion of a quiescent, demethylated gene (gamma-globulin or hepatitis B core antigen) to an active state requires some endogenous or exogenous inducing agent, which may be highly specific for any given gene or gene complex. Clonal selection is not responsible for observed changes in gene expression, since we have clearly shown that cells remethylate DNA that has been demethylated by 5-azacytidine treatment.

New micro techniques have been developed which enable the analytical quantitation of 5-methylcytosine in less than 1 μ g of DNA isolated from any source. Thus, the genomic 5-methylcytosine content of normal human bronchial epithelial and pulmonary mesothelial cells has been measured for the first time.

Somatic Cell Genetics of Human Lung Cancer Studied by Cell Hybrids

Somatic hybrids of human cells are being prepared and analyzed to study the genetics of human lung cancer. This project is focusing on the following cells as parents for hybridization: 1) normal human bronchial epithelial cells; 2) human lung carcinoma cell lines (H292 and A1146); 3) bronchial epithelial cell lines that have been transfected with the v-Ha-ras gene, are apparently immortal in culture, and exhibit a varying degree of tumorigenicity in athymic nude mice (TBE-1 and TBE-SA), and 4) a mouse-human hybrid cell line that has a single human chromosome 11 to which has been translocated a segment of the X-chromosome containing the HPRT locus (X-11). Preliminary experiments have found that hybrids of normal cells with H292 cells are all mortal in culture. Hybrids of TBE-1 with H292 are frequently immortal, and these cell lines are being examined for tumorigenicity in athymic nude mice.

Human Chorionic Gonadotropin as a Marker and Growth Factor in Human Lung Tumors

We have immunohistochemically examined a large number of normal, hyperplastic, metaplastic, and neoplastic tissues to detect the presence of β -human chorionic gonadotropin. The hormone was present in normal fetus and placenta and in more than 75% of adult lung neoplasms [except small cell lung carcinoma], but not in normal adult tissues. It was also synthesized in tumors generated in athymic nude mice by v-Ha-ras transformed bronchial epithelial cells.

Proliferation of Normal Human Mesothelial Cells and of Mesotheliomas

We have determined that transforming growth factor type beta (TGF- β) is a mitogen for normal human mesothelial cells which do not produce this protein. In contrast, a mesothelioma cell line has been shown to produce TGF- β . Neither normal nor asbestos-modified mesothelial cells form tumors in athymic nude mice, while two mesothelioma lines tested do show this oncogenic potential. A model of autocrine stimulation and/or maintenance of mesothelioma cells by TGF- β is suggested.

IV. CARCINOGENESIS AND RELATED STUDIES

A central problem of cancer research is the extrapolation of carcinogenesis data and knowledge of carcinogenesis mechanisms from laboratory animals to humans and, within this heterogeneous population, extrapolation among individuals. An aspect of this problem is the difficulty associated with extrapolating from one level of biological organization to another, i.e., from molecules to macromolecules to organelles to cells to tissues to intact organisms. Multiple experimental systems are needed to help investigators find solutions to these and other problems in carcinogenesis research. Animal models are obviously required for experimental in vitro carcinogenesis studies. They are also essential because the integral multisystemic interactions of the organism remain intact and because laboratory animals can be environmentally and genetically controlled. In vitro models using tissues, cells, and subcellular fractions are also useful. This approach can aid in the resolution of the central problem of extrapolation in that one can conduct comparative studies with tissues and cells from experimental animals and humans that are maintained in the same controlled in vitro experimental setting. Carcinogenesis studies using human tissues and cells offer unique opportunities. For

example, some rare forms of human cancer reflect inherited, predisposing conditions, and their genetic basis and perhaps common pathways of carcinogenesis may be understood through the study of cells from individuals with these specific types of cancer. In addition, because human cells in vitro are apparently genetically more stable and undergo less "spontaneous" neoplastic transformation than most rodent cells, they may be especially suitable for studying the multistage process of carcinogenesis.

Epithelial cells are of particular interest because most adult human cancers are carcinomas. As noted above, significant progress has been made in the past decade in developing methods for culturing human epithelial tissues and cells. Chemically defined media have been developed for culturing normal human tissues and cells from organs with a high rate of cancer in humans. Serum-free media have several advantages in studies of cultured human cells, including (a) less experimental variability compared to serum-containing media, (b) selective growth conditions of either normal cells of different types (e.g., epithelial versus fibroblastic cells) or normal versus malignant cells, (c) identification of growth factors, inhibitors of growth, and inducers of differentiation, and (d) ease of isolating and analyzing secreted cellular products. Advances in cell biology, including the delineation of biochemical and morphological markers of specific cell types, have also facilitated the identification of cells in vitro (e.g., keratins as markers for epithelial cells and collagen types I and III for identifying fibroblasts).

Carcinogenesis is a multistage process that can be operationally divided into tumor initiation, promotion, conversion, and progression. Genetic changes, perhaps mutations, are considered to be responsible for tumor initiation and malignant conversion. As was noted earlier, metabolism of carcinogens, DNA damage, and DNA repair are considered to be important factors in these stages of carcinogenesis.

Oncogene Transfection of Human Bronchial Epithelial Cells

Genetic studies of human cell DNA repair and carcinogenesis have been initiated by protoplast fusion transfection of a variety of types of human cells with specific cellular and viral genes known to play a role in human carcinogenesis. The role of altered ras genes in normal human bronchial epithelial (NHBE) cell carcinogenesis is being investigated by studying v-Ha-ras transfected NHBE cells. The protoplast fusion method was used to transform NHBE by transfection with the pBR322/Ha-MSV plasmid carrying the v-Ha-ras gene (H1). The rescue of episomal forms of v-Ha-ras DNA from the transformed human cell line (TBE-1) isolated by transfection of TBE-1 DNA preparations into E. coli followed by selection for Ap^R infers that the transfected plasmid recombined with naturally occurring episomal elements to form a "shuttle-plasmid" capable of replication in human cells and in E. coli. The presence of v-Ha-ras on approximately 80%, and human Alu-sequences on 100%, of the plasmids recovered in this fashion is consistent with the "shuttle-replication" properties required for stable maintenance of transfected DNA in human cells when these genetic elements are covalently linked to pBR332 and other DNA fragments for transfection analysis.

Characterization of v-Ha-ras-transfected NHBE cells, including restriction mapping of integration loci, episomal Ha-ras DNA, selection of tumorigenic and

nontumorigenic clones, and determination of conditions required for expression of tumorigenic phenotypes, will provide information to elucidate the mechanism of ras-mediated carcinogenesis in an important progenitor cell of human lung cancer.

Transfection of Oncogenes into Normal Bronchial Epithelial

Normal human bronchial epithelial (NHBE) cells have been successfully transfected with the oncogenes raf, a combination of raf and v-myc and a translocated c-myc gene isolated from the Burkitt's lymphoma cell line CA46. The oncogenes transfected were cloned into pSV_{2neo} allowing selection for G418 resistance and were expressed from a viral promoter.

Transfected clones were selected by taking advantage of the fact the transformed cells are resistant to inducers of differentiation like blood-derived serum or the tumor promoter 12-O-tetradecanoylphorbol-13-acetate. Among the three oncogenes analyzed by transfection into NHBE cells, the translocated c-myc gene was the most potent inhibitor of differentiation in NHBE cells. In order to analyze the elements of this translocated c-myc gene that are critical for the regulation of differentiation, deletion mutants of the original clone were made which contained deletions of c-myc, the immunoglobulin heavy chain, or the switch region.

Isolation and Characterization of Homologs of the Raf Oncogene

We have isolated the raf homologs or related genes from man, mice, birds, fruit flies, and yeast. DNA sequence of the conserved kinase domain is available for all but the yeast homolog. The predicted amino acid sequences reveal that human and avian genes are 95% homologous to the murine gene, while the Drosophila shares only 65% of its sequence with the mouse. The most invariant region is the 200+ amino acid group from the nucleotide binding domain (-G-X-G-X-X-G-13 amino acids-K-) to the sequence -M-V-G-R-G-, which is probably also an element of the nucleotide binding activity. Expression of the Drosophila homolog (D-raf-1) is limited to the late stage of embryonic development. This gene has been localized to the X chromosome, more specifically the 2F 5, 6, region. Within the 2F band, four complementation groups have been described, all of which when mutant are lethal during embryonic development. Observation of two raf-related sequences in Drosophila has led to the isolation of a new human raf-related gene that has been localized to the short arm of the X chromosome. Attempts to recognize the raf homolog in yeast were unsuccessful until the D-raf-1 sequence was used as a probe. The yeast homolog has been cloned into a pBR322 vector, and subcloning is in progress.

The Characterization of a New Human Proto-Oncogene (PKS) Related to the raf Subfamily of Oncogenes

A human fetal liver cDNA library was screened at reduced stringency for v-raf-related sequences. In addition to the expected c-raf-1 cDNA, a novel sequence was isolated. Comparison of the new gene (pks-1) to the other raf homologs revealed nucleotide homologies of 71%. The predicted amino acid sequence of the kinase domain is sufficiently similar to that of v-raf to suggest that pks-1 is a receptor that exhibits serine/threonine kinase activity. The expression of pks-1 mRNA (2.8-kb) is elevated in peripheral blood mononuclear

cells isolated from patients with systemic lupus erythematosus and angio-immunoblastic lymphadenopathy with dysproteinemia (AILD), two diseases in which autoantibodies are produced following the lymphoproliferative activation of B cells. In the course of localizing the pks-1 gene to the short arm of the X chromosome (Xpter-Xp11), a related gene (pks-2) was recognized and localized to chromosome 7 (7pter-7q22). The role of the pks-1 gene in X-linked immunodeficiency and lymphoproliferative diseases is being investigated.

The Expression of the Raf Oncogene in Human Malignancies and in Lymphoproliferative Diseases

We have investigated the expression of the c-raf-1 proto-oncogene in neoplastic as well as hyperplastic pathologies. All small cell lung carcinomas examined expressed elevated levels of raf transcripts; this was particularly true of the non-cultured metastases. Characterization of surface markers revealed the presence of early monomyelocyte antigens, indicating that these cells can express phenotypic traits of hemopoietic precursors. Activated T and B cells obtained from the peripheral blood of human autoimmune-diseased individuals or lymph nodes and spleens isolated from autoimmune mice also expressed elevated levels of the raf proto-oncogene transcript. In both cases, the gene appears structurally unaltered, suggesting that the defect lies in a transcriptional controlling element(s).

Model Systems for Studying Physical Carcinogens at the Cellular Level

Our laboratory is using cultured normal human bronchial epithelial cells, human bronchial fibroblasts, and human pleural mesothelial cells to study carcinogenesis induced by asbestos and related fibers at the cellular level. Electron microscopy studies demonstrate that asbestos fibers first attach to the cell membrane and are then phagocytosed into the cell. Even though all three cell types exhibited equivalent numbers of internalized fibers 24 hours post-exposure to the same concentration of amosite, the pattern of cytotoxicity to fibers in culture was found to parallel the selectivity of cell types as targets for carcinogenesis in vitro, e.g., asbestos and glass fibers were found to be 100 times more cytotoxic for the mesothelial cell and 10 times more cytotoxic for the bronchial epithelial cells than for the bronchial fibroblasts.

Multiple studies failed to establish a role of free radicals in asbestos cytotoxicity and carcinogenesis: 1) electron paramagnetic resonance (EPR) using the spin trap molecule 5,5-dimethyl-1-pyrroline-1-oxide (DMP0) failed to detect the production of oxygen radicals in mesothelial cells exposed to asbestos; 2) free radical scavengers (superoxide dismutase, glutathione, N-acetylcysteine, and D- α -tocopherol) failed to modify the cytotoxicity of asbestos fiber cytotoxicity; and 3) alkaline elution of DNA extracted from mesothelial cells exposed to asbestos fibers failed to detect an increase in single strand breaks. Other fibers were also examined for cytotoxic effects on human mesothelial cell cultures. Code 100 thin glass fibers are similar in toxicity to amosite asbestos fibers, but samples of code 110 thick fibers and carbon graphite fibers taken from workplace settings are much less toxic.

Focal hyperplasia and epidermoid metaplasia were observed in explants of human bronchial tissue 2 weeks after a single exposure to amosite asbestos; both

intracytoplasmic and intranuclear asbestos fibers were seen by X-ray microanalysis in these lesions. However, these atypical lesions proved to be transient and were not detected in tissue examined 12 weeks post exposure. Normal human bronchial cells were also exposed to amosite, but no abnormal cells were recovered from these cultures. On the other hand, colonies of phenotypically transformed mesothelial cells arose in cultures two subculturings after amosite exposure. These amosite-altered cultures continued to multiply for more than 18 subculturings, whereas the control cultures stopped growing during the fourth subculture. The exposed cells had a near-normal modal number of chromosomes through the sixth subculturing. At the ninth subculture, 80% of the metaphases had dicentric chromosomes, and the modal number had increased to 77.

Model Systems for Studying Nickel-Associated Bronchial Epithelial Cell Carcinogenesis

Cultures of normal human bronchial epithelial cells were continuously exposed to a dose (5-20 $\mu\text{g/ml}$) of NiSO_4 that reduced their colony-forming efficiency by 30-80%. After 40 days of incubation, the cultures consisted of large squamous cells; mitotic cells were not observed. The cells were then maintained in medium without NiSO_4 . After 40 to 75 total days of incubation, colonies of mitotic cells appeared at a rate of 1 colony per 100,000 cells originally at risk; no colonies appeared in control cultures or in cultures exposed to $< 5 \mu\text{g NiSO}_4/\text{ml}$ for 90 days. Twelve NiSO_4 -altered cell cultures isolated from five experiments were expanded into mass cultures. None of the isolates are immortal, but most of the lines have an increased population doubling potential (>50 divisions). Some exhibit aberrations in the squamous (terminal) differentiation process, whereas others have lost the requirement for epidermal growth factor for clonal growth. Aneuploidy and marker chromosomes have also been noted. However, none of these NiSO_4 -altered cell cultures are anchorage independent, nor do they produce tumors upon injection into athymic nude mice.

Hepatitis B Virus Carcinogenesis

The protoplast fusion method for transfection of human cells was used to construct a human epithelial cell line carrying the hepatitis B virus (HBV) core gene (HBc) stably integrated into the genome (GTC2). The plasmid pKYC200 used to construct GTC2 contains the HBc structural gene and its regulatory elements without other HBV genes. Therefore, GTC2 cells provide an *in vitro* model system to determine the effects of HBc gene expression without interference from other HBV genes. The cytopathological effects observed during the maximal expression of HBc gene product provide an insight into the role HBc regulation plays in the pathology of HBV infection. The regulation of HBc expression by the methylation of an Hpa II site 280 bp upstream of the AUG encoding the start site of the HBc structural gene provides the first proof for site-specific regulation of a human virus gene by methylation of 5'-methylcytosine. In addition, the regulation of HBc gene expression during the treatment of cells in culture with alpha interferon indicates that the expression of HBc may be regulated both at the transcriptional and translational levels since demethylation of the Hpa-280 site not only increases transcription but also increases the level of gene product, although the level of HBcAg does not, suggesting that the transcripts are not appropriated for translation.

Genetic Structure and Regulation of RNA Tumor Virus Expression

We have utilized a pol mutant of murine leukemia virus to further define the functional domains of the mammalian pol gene. We have shown that the mutant contains only one biologically significant alteration and we have localized and sequenced the change. This alteration results in immediate termination of translation, since a stop codon is brought immediately into frame. We have phenotypically characterized this mutant to show that gag proteins are present and cleaved normally and that a truncated reverse transcriptase molecule of 50 kd is synthesized. This enzyme contains reverse transcriptase and RNase H activities at severely reduced specific activities but is able to normally initiate synthesis and transfer from the 5' to the 3' end of genomic RNA. The mutant enzyme cannot, however, read past regions of the template, which appear to be pause sites for wild-type enzyme. The size of the mutant reverse transcriptase molecule and precise knowledge of its C terminus allow us to conclude that the murine leukemia virus pol gene contains coding capacity for a protein of about 13 kd upstream of reverse transcriptase and a protein of about 40 kd downstream of the polymerase coding region. The 13 kd region must be the viral-coded protease, since this activity is present in the mutant. Furthermore, the RNase, H and DNA polymerase active sites must be localized to the aminoterminal two-thirds of the reverse transcriptase molecule.

In addition, we have selected two Moloney murine sarcoma viruses (MuSV) of differing phenotype but similar restriction maps for DNA sequencing. The sequencing, now completed, suggests that critical signals for translation of the major structural genes lie in the 5' untranslated portion of the genome and that a deletion as small as 48 bp including the major 3' splice acceptor allows only very low expression of the mos oncogene through use of alternative splice acceptors.

In Vitro Human Epithelial Carcinogenesis Studies Utilizing Human Epithelial Cell Culture Systems

We have transformed human epidermal cells in vitro by DNA transfection using subgenomic fragments of DNA from wild-type SV40 virus or a temperature-sensitive A mutant (tsA209). The SV40-transformed cells differed from non-transformed cells in their morphological appearance, growth properties, and the expression of certain characteristics associated with differentiation, namely keratin proteins and cross-linked envelopes. In general, transformation of the epithelial cells was associated with a loss of epithelial cell-specific properties. Expression of these functions was restored at the non-permissive temperature (40°C) in the tsA209 transformed cells, demonstrating the dependence of these functions on a functional A gene product (i.e., a functional SV40 transforming protein). The cells are immortalized and can grow in an anchorage-independent fashion (late passaged cells), but as yet, are non-tumorigenic unless transfected with other viral DNA, such as human papillomavirus.

Model Systems for Studying Tumor Promotion in Human Bronchial Epithelial Cells

Because there is epidemiological evidence that tumor promotion plays a role in the development of human bronchogenic carcinoma, we are investigating the effects of putative tumor promoters on normal human bronchial epithelial (NHBE) cells. The mouse skin tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) induces markers of terminal squamous differentiation in NHBE cells, including a squamous morphology change, increased plasminogen activator activity, cross-linked involucrin envelope (CLE) formation, and cessation of proliferation. In contrast, lung carcinoma cell lines are relatively resistant to the effects of TPA. These findings are consistent with the hypothesis that one mechanism of tumor promotion involves resistance of preneoplastic and neoplastic cells to inducers of terminal differentiation. Compounds representing two other classes of mouse skin tumor promoters, the polyacetates and the indole alkaloids, also induce terminal differentiation. Because cigarette smoke condensate (CSC) contains mouse skin tumor promoters, we studied the effects of CSC and fractions thereof on NHBE cells. None of the fractions was mitogenic over a broad range of concentrations. All of the fractions inhibited proliferation, with the most potent being the neutral methanol fraction (N_{meOH}). N_{meOH} also induced plasminogen activator activity, had the most pronounced effects on CLE formation, and was the most potent inhibitor of specific binding of epidermal growth factor. Based on these findings, we concluded that the N_{meOH} fraction is the most likely to contain compounds with effects similar to those of the mouse skin tumor promoter.

V. BIOCHEMICAL AND MOLECULAR EPIDEMIOLOGY

The primary goal of biochemical and molecular epidemiology is to identify individuals at high cancer risk by obtaining pathobiological evidence of (1) high exposure of target cells to carcinogens and/or (2) increased host susceptibility due to inherited or acquired factors. Laboratory methods have been developed recently to be used in combination with analytical epidemiology to identify individuals at high cancer risk. These methods include (1) techniques to assess specific host susceptibility factors; (2) assays that detect carcinogens in human tissues, cells, and fluids; (3) cellular assays to measure pathobiological evidence of exposure to carcinogens; and (4) methods to measure early biochemical and molecular responses to carcinogens.

Currently available techniques exist that would allow the utilization of biochemical and molecular measures to better characterize exposure to carcinogens, to serve as intermediate end points on the path to malignancy, to identify measures which halt or reverse this process, and to investigate the mechanisms of human carcinogenesis. Included in these investigations would be the following: (1) efforts to evaluate body burden of chemical carcinogens in studies of occupational and general environmental cancer risk factors; (2) sophisticated analyses of air, water, and biological specimens for carcinogenic and mutagenic substances in conjunction with specific analytical studies; (3) search for evidence of viral infection including viral segments or oncogenes in the DNA of individuals at high risk of cancers that may be associated with infectious agents or heritable states; (4) evaluation of disturbances in immune function as they may relate to malignancies, particularly those of the hematopoietic system; (5) investigation of the relationship between micronutrients and a variety of epithelial cancers; and

(6) determinations of the relationship between macronutrients, including dietary fat and subsequent hormonal changes, to subsequent risk of breast, endometrial, and perhaps colon cancer.

Measurement of Carcinogen-DNA Adducts and Antibodies to These Adducts in Humans and Experimental Animals

Benzo[a]pyrene (BP) is a ubiquitous carcinogen found in tobacco smoke, burning fossil fuels, and our diet. Formation of BP diol epoxide (BPDE)-DNA adducts due to human exposure is most likely to be at very low levels that are beyond the sensitivity of routine radioimmunoassay and chromatographic analyses. Thus, ultrasensitive enzymatic radioimmunoassay (USERIA), enzyme-linked immunosorbent assay (ELISA), and synchronous scanning fluorimetry have been employed to detect and quantitate BPDE-DNA adducts in humans at high cancer risk due in part to BP exposure. DNA isolated from white blood cells of asphalt workers (roofers) and foundry workers and DNA from lung tissue, bronchial washings, and alveolar macrophages of lung cancer patients and smokers are being investigated. Putative BPDE-DNA adducts have been detected in several of the high-risk individuals. Antibodies to BPDE-DNA adducts were also found in sera from those workers. These data suggest that the activation of BP to its ultimate carcinogen as well as formation of adducts with DNA occurs in humans. Preliminary evidence indicates that other polycyclic aromatic hydrocarbons, e.g., chrysene- and benzo(a)anthracene-DNA adducts, may also evoke an immune response in humans.

Analysis of Hydrocarbon-Macromolecular Adducts in Humans; Relation to Cancer Risk

Polycyclic aromatic hydrocarbons (PAHs) are frequently encountered environmental contaminants, which have been shown to be metabolized *in vivo* to reactive intermediates (e.g., benzo[a]pyrene-diol-epoxide BPDE) that bind covalently to cellular macromolecules (DNA and protein). It is postulated that the detection of PAH-DNA and PAH-protein adducts could be used as dosimeters for PAH-exposure in human populations.

BPDE-DNA adducts were prepared synthetically and subjected to analysis by spectrophotometry, spectrophotofluorimetry and ultrasensitive-enzyme-linked-radioimmunoassay (USERIA). The extent of DNA modification was originally determined to be 1% (one adduct in 100 nucleotides) using the method of Pulkrabek (Biochemistry 16: 3127, 1977). It was further determined that one adduct in 1.4×10^7 nucleotides could be detected using spectrophotofluorimetry and one adduct in 2.8×10^7 nucleotides could be detected by USERIA. Both of these methods were subsequently applied to the analysis of human-DNA samples that were obtained from individuals who were occupationally and/or self-exposed (cigarette smoking) to PAHs. Among a group of coke oven workers, the presence of hydrocarbon-DNA adducts was detected in 31 out of 41 (76%) individuals by spectrophotofluorimetry and in 18 out of 27 (67%) by USERIA. DNA-adducts that have been prepared from benz[a]anthracene-8,9-diol 10,11-oxide and chrysene-1,2-diol 3,4-oxide are currently being characterized by fluorescence spectroscopy. It has been possible using a combination of high-performance liquid chromatography and synchronous fluorescence spectroscopy to detect and distinguish between three types of PAH-DNA adduct that were present in a relatively simple synthetic mixture.

Synchronous Fluorescence Scanning Detection of Aflatoxin Adducts

Synchronous fluorescence scanning (SFS) and computer linkages for data analysis have been used to characterize the fluorescent properties, limits of detection, contour maps, and three-dimensional images of a series of aflatoxins, their metabolites, and DNA adducts. The power of these fluorescence techniques has been shown by the demonstration that the number of components within a sample can be delineated using fourth derivative computer analysis of three-dimensional SFS data. Thus, these techniques may provide valuable structural determinations of fluorescent carcinogens and DNA adducts.

Immunobiology of HTLV-I

The human T-cell leukemia lymphoma virus (HTLV-I) is a human retrovirus that was isolated by Gallo and associates from a cell line originating from a patient with adult T-cell leukemia. Over the past 4 years, epidemiological studies have clearly suggested that this agent is causative for adult T-cell leukemia. In studies of the T- and B-cell lines established from the individual from which the virus was originally isolated, alteration in expression of HLA-A and -B antigens was observed in the infected T-cell line and not in the HTLV-I-negative B-cell line. Studies demonstrated that HTLV-I could be transmitted to and isolated from cord blood lymphocytes. The cell surface antigen changes that were associated with infection were the increased expression of HLA-DR, the T-cell growth factor receptor (TAC), and altered HLA antigen expression, as determined by alloantisera and a monoclonal antibody 4D12. This monoclonal antibody detects an epitope in HTLV-I-infected cells is independent of the human chromosome 6. Alteration in immune function has been observed in patients with HTLV-I-related diseases. This clone line had cytotoxic activity against other HTLV-I-infected cell lines which was restricted by the HLA-A1 antigenic determinant. These results give some insight into the control of the disease. The restricted cytotoxic function was shown in antibody inhibition studies to be related in part to the cross-reactive HLA class I epitope (4D12), which is expressed on the cell surface of HTLV-I-infected cells. B-cell lines established as spontaneous outgrowth of cultured cells from two ATL patients with HTLV-I-infected leukemic T-cells were the first demonstration that B cells could be infected with the HTLV-I retrovirus. Co-culture of these B-cell lines with peripheral blood lymphocytes or cord blood lymphocytes resulted in infection of T-cells and not B-cells, demonstrating that the virus had not altered its selective tropism for T-cells. To better understand the structural proteins of HTLV-I and their relationship to immunopathogenic events, a shuttle vector was constructed using the bovine papillomavirus and a portion of the HTLV-I retrovirus. Monoclonal antibodies developed by immunization of cells transfected with the shuttle vector recognize the small envelope protein (P21) of this virus. The observation that HTLV-I endemic areas suggested that HTLV-I infection may also indirectly serve as the etiologic agent for B-cell neoplasms. In other studies, we demonstrated that we could capture immunoglobulins from chronic lymphocytic leukemia (CLL) cells by fusing these cells with the human lymphoblastoid cell line. Experiments were performed in two patients with CLL whose sera contained antibodies to HTLV-I but lacked HTLV-I infection in the CLL cells. Immunoglobulins captured from these cell lines had antibody activity to HTLV-I retrovirus proteins. This is the first demonstration that a B-cell malignancy is antigen committed, probably prior to events that result

in malignant transformation. Furthermore, one of the monoclonal antibodies developed from this experiment demonstrated cross-reactivity to epitopes on the gag proteins from HTLV-I, II, and III.

HLA Antigens, Structure, Function and Disease Association

For the past four years, we have investigated and reported the results of studies of HLA typing of populations of individuals and families with common or related diseases. These studies were performed in an attempt to see if there was a specific association of a particular HLA phenotype with the disease and whether or not such phenotypes were represented in families in which more than one individual had a common disease entity. HLA haplotype associations were found in family members with 17-hydroxylase deficiency, both the congenital form and the milder enzymatic defect which is manifest at the time of puberty. A population of individuals with Takayasu's arthritis were HLA typed and found to have an increased frequency of HLA-DR4 and MB3. It was previously demonstrated that HLA-B27 had a high degree of association with sacroiliitis in Caucasian populations. HLA typing of the American Indians with this disease also showed this same association. HLA antigen frequencies were studied in the Gila River Indians, including the Pima and Papago Tribes. An extreme genetic restriction in HLA class I and class II antigens was found in these populations. In addition, it was found that these two closely related tribes (geographically and historically) maintained certain HLA phenotypes which are specific for each tribe. HLA antigen frequencies were studied in a population of individuals with rheumatoid arthritis, a disease where an HLA-DR4 association has been demonstrated. We observed that certain combinations of HLA-A, B, and HLA-DR antigens were more frequent in the diseased individuals compared to these same combinations in the normal population. This apparent association between a number of antigens and the disease suggests that a combination of genes closely linked in the major histocompatibility complex (MHC) 6.

HLA typing was performed in a population of individuals with systemic lupus erythematosus and in families where one or more members had this disease. Increased frequency of HLA-DR2 and an associated MT1 determinant was found in the disease population as well as in family members with this disease.

Sub-populations of patients with Sjogren's syndrome with different autoimmune features of this disease demonstrated specific HLA-DR antigen combinations with each autoimmune subset. In families with Hodgkin's disease, homozygosity of the HLA-DR MT1 locus was observed more frequently in cases in families than was observed in the non-diseased family members. HLA typing was performed in seven families where multiple individuals either had the pre-malignant mole syndrome or malignant melanoma. In contrast to other reports, no definite association with the major histocompatibility complex was observed in this family study. Families exposed to the HTLV-I retrovirus were studied for HLA antigen phenotypes and haplotypes. These families included at least one member who had ATL and other family members who had antibody to the HTLV-I retrovirus. There was no specific HLA association with the disease ATL; however, in those individuals who had ATL and/or antibody to the retrovirus, an HLA association was observed in some families. The lod score in all the families was 1.2.

The HLA-DR region of the MHC has been postulated as a regulatory region for the immune response. We studied the genetic control of immune response to insulin, an obligatory therapeutic modality in insulin-dependent diabetics, and a potent immunogen. Lymphocyte stimulation with various therapeutic insulin compounds was used as a measure of the immune response. Specific response to beef insulin was associated with DR4, beef and pork insulin with HLA-DR3, and protamine with HLA-CW3, and CW5, and DR7.

HLA-DR expression in newborn cord blood lymphocytes was studied. It was found that one could induce expression of HLA-DR antigens with various lymphokines, particularly gamma interferon.

The control of HLA-DR expression was studied in cell lines derived from patients with adult T-cell leukemia and childhood acute lymphocytic leukemia (ALL). The former express HLA-DR antigens and are infected with HTLV-I, while the latter are not infected with HTLV-I and express no HLA-DR antigens. Phorbol esters induce expression of HLA-DR antigens and demethylation of HLA-DR genes in all ALL-derived T-cell lines. In short-term culture of fresh lymphocytes, adult T-cell surface markers occur at the same time as expression of viral proteins. The HLA-DR genes were found to be methylated to the same extent as the fresh adult T-cell leukemias. The results suggest that the HTLV-I retrovirus induces or controls HLA-DR expression at a post-transcriptional level.

Alloantisera and monoclonal antibodies to the HLA-DR determinants were found to interfere with antigen processing and subsequent stimulation of T-cells. In examining the mechanism for this, we observed that certain antibodies reacting with HLA-DR on monocytes (antigen-presenting cells) selectively induced activation of suppressor T-cells. This suppressive mechanism was abrogated by removal of the Fc piece from the antibodies to HLA-DR.

Structural studies of the products of the HLA-DR region were performed to determine the relationship to the multiple serological determinants expressed on B cells. Using homozygous cell lines that expressed HLA-DR and related antigens and antisera reacting with these antigens, it was determined that the MT2-MB3 and MB4-bearing molecules had patterns of α - and β - chain mobility in two-dimensional gel electrophoresis that differed from HLA-DR5 molecules. These early studies strongly suggested there was more than one locus in the D region which controlled the expression of HLA-DR-related antigen determinants. It was further demonstrated that there were three structurally distinct human HLA-DR beta chains by two-dimensional electrophoresis. Other investigators have subsequently demonstrated that there are three distinct loci which control the expression of heavy and light chains of the HLA-DR locus.

Epidemiologic and Immunobiologic Studies of the Acquired Immunodeficiency Syndrome (AIDS)

The acquired immune deficiency syndrome (AIDS) was first described by Gottlieb et al. in 1981. This disease is characterized by a profound immunodeficiency as well as the neoplastic disease Kaposi's sarcoma. One of the features of this disease is the deficiency of helper T lymphocytes which can be defined by the antibodies detecting the T4 molecule. We have examined the numbers of T4⁺ cells and suppressor cells (defined by the monoclonal antibody detecting the

T8 antigen) in peripheral blood lymphocytes from patients with AIDS and those at risk for this disease. In our initial investigation, we examined these T-cell markers in a population of homosexuals and patients with Kaposi's sarcoma. A decreased number of $T4^+$ lymphocytes and thus a low $T4/T8$ ratio was found in these individuals and appeared to correlate with the use of amyl and butyl nitrite. The studies were extended to investigate a group of Danish homosexuals where it was observed that approximately one-third had low helper ($T4^+$) suppressor ($T8^+$) T-cell ratios. In this study, there was no correlation with nitrite use. The only positive risk factor for decreased $T4/T8$ ratios was sexual contact with individuals in the United States. Studies were also conducted using lymphocytes from male homosexuals from the Washington, D.C. area. In this study, potential risk factors, such as sexual contacts and practices, were correlated with numbers of $T4^+$ lymphocytes in peripheral blood specimens. The lowest numbers of $T4^+$ cells were found in Washington D.C. homosexual males who had sexual contact with high-risk groups, i.e., individuals from the New York or San Francisco areas. Normal numbers of $T4^+$ cells were found in homosexuals (defined as low risk) who had had no contact with individuals from the high-risk group. Intermediate numbers of $T4^+$ cells were found in those individuals that had had sexual contact with the Washington, D.C. high-risk group. In comparing sexual practices with numbers of $T4^+$ -positive lymphocytes, the number of sexual partners correlated with the lower $T4^+$ cells, as did receptive anal intercourse. These studies inferred the possibility that there was a transmissible agent that was responsible for this disease. Gallo and his colleagues subsequently isolated a retrovirus that is now known as HTLV-III. Assays for antibodies to this virus were established, and we investigated the frequency of antibody positivity in individuals who had AIDS or who were at risk for this disease. The studies showed that there was a close relationship between low numbers of $T4^+$ cells and HTLV-III zero-positivity. In addition, we found that antibody positivity was more frequent in individuals with a large number of homosexual partners as well as in those individuals who participated in receptive anal intercourse. In vitro depletion of $T4^+$ -positive lymphocytes was studied using peripheral blood lymphocytes (PBL) from heterosexual males, homosexual males, and patients with AIDS. PBL were cultured in the presence of a T-cell mitogen, PHA, and acid-stable and acid-labile α -interferon. These studies showed that mitogenic stimulation (PHA) together with alpha interferon caused a decrease in the number of $T4^+$ cells in HTLV-III seropositive individuals. We investigated the possibility that the HTLV-III retrovirus binds specifically to a T-cell molecule which bears the T4 antigenic determinant. In these studies, we demonstrated that the specific binding site is on a portion of the molecule which is recognized by monoclonal antibodies detecting epitopes other than the T4 determinant. Two monoclonal antibodies, OKT4D and OKT4F, lost their binding capacity to the cells within 10 minutes of incubation with HTLV-III virus. The epitope detected by the OKT4 monoclonal antibody was unaffected during this period of time. After 48 to 72 hours, the determinant detected by the OKT4 antibody disappeared and the HTLV-III infection of the cell was demonstrated. We interpret these data to suggest that this molecule is the specific receptor site and that the molecule is internalized as a process of HTLV-III infection.

CONTRACT IN SUPPORT OF THESE PROJECTS: Z01CP05191-06 LHC, Z01CP05193-06 LHC, Z01CP05291-05 LHC, Z01CP05293-05 LHC, Z01CP05324-04 LHC, Z01CP05325-04 LHC, Z01CP05341-04 LHC, Z01CP05403-03 LHC, Z01CP05409-03 LHC, Z01CP05410-03 LHC, Z01CP05424-02 LHC, Z01CP05426-02 LHC, Z01CP05427-02 LHC, Z01CP05429-02 LHC, Z01CP05431-02 LHC, Z01CP05432-02 LHC, Z01CP05433-02 LHC, Z01CP05435-02 LHC, Z01CP05477-01 LHC, Z01CP05478-01 LHC, Z01CP05479-01 LHC and Z01CP05480-01 LHC

UNIVERSITY OF MARYLAND (N01-CP-51000)

Title: Collection and Evaluation of Human Tissues and Cells from Patients with an Epidemiological Profile

Current Annual Level: \$474,363

Man Years: 6.65

Objectives: To provide a resource to the NCI for the procurement, transport, and characterization of normal, preneoplastic, and neoplastic human bronchus, pancreatic duct, colon, and liver from patients with an epidemiological profile.

Major Contributions:

1. Collection of Tissues

Tissue specimens were collected from a total of 177 cases at this writing, including specimens from surgery patients (94) with and without cancer and at time of autopsy (83). Autopsy specimens are collected from patients undergoing either immediate autopsy (5) (i.e., within 30 minutes after death) or routine autopsy (78) (i.e., between 2 and 12 hours after death). All tissues received at the NIH are usually residuals of materials taken for regular diagnostic and corrective purposes and not for research per se.

A. Surgical Specimens

A total of 94 surgeries resulted in tissue donations.

Bronchus: Tumor tissues from 59 cases of lung carcinoma were collected. Bronchial specimens uninvolved with tumor were provided from 58 of these cases and transported to the NIH. Twelve specimens of lung tumor were received. The tumors were defined and classified as described below.

Colon: Tumor tissues from 35 cases of colon carcinoma were collected. Colonic tissue uninvolved with tumor from 53 cases (43, UMH; 10, VAHLR) was transported to the NIH. All of the tumors were defined and classified as described below.

3. Definition and Classification of Nonneoplastic and Neoplastic Tissue

Epidemiologic data are provided to allow determination of the relationships between tumor type and selected risk factors and the amount of benzo[a]pyrene (BP) or aflatoxin B₁ (AFB), respectively (e.g., in lung cancer and hepatoma), bound to DNA by the same patient's noncancerous epithelium.

Bronchus: Morphological and histochemical characterization of human primary lung carcinomas are routine. Characterization of tissues by immunocytochemistry has continued using the peroxidase-antiperoxidase method to demonstrate the presence or absence of various antigens. Tumor and normal tissues, abnormal and preneoplastic tissues are examined for beta human chorionic gonadotropin (HCG), calcitonin, adrenocorticotrophic hormone (ACTH), serotonin, alphafetoprotein (AFP), keratin, somatostatin, neuron specific enolase (NSE), calmodulin, and tubulin.

Normal and abnormal (but non-neoplastic) adult bronchi contain only mucosubstances, keratin, calmodulin, and tubulin. Keratin is seen in normal bronchial epithelium (including bronchial glands) and occasionally in columnar cells that reach the lumen. Calmodulin apparently increases levels at the cell borders.

Lung tumors have greater heterogeneity than tumors in the bronchial epithelium. Each marker is found at least occasionally in tumor, HCG is found in 80% of non-small cell tumors, and keratin is found in 75% of such tumors. Somatostatin is seen in keratinizing areas and more diffusely in a smaller proportion of adenocarcinomas. NSE and serotonin are seen in endocrine tumors only, and only in tumors with dense-cored granules, including small cell carcinomas, carcinoids and atypical endocrine carcinomas. Appearance of other markers (observed less commonly--ACTH, 40%; somatostatin, 40%; calcitonin, 20%; AFP, 2%) shows less predilection for types of lung tumors, and HCG seems to follow glycogen distribution.

Indirect immunofluorescent detection of tubulins is performed on cellular outgrowths of bronchial explants involved and uninvolved with tumor. Generally uniform, the normal cells have mostly straight microtubules originating from assembly sites near the nucleus, while the variable tumor cells had irregular microtubular patterns in a mesh-like arrangement.

Colon: Morphological (light microscopy [LM], transmission electron microscopy [TEM] scanning electron microscopy [SEM]) and histochemical examinations of normal premalignant and malignant human epithelium are routine. The comprehensive description of the morphology of normal human colon is still incomplete. In ascending segments, apical vesicles are EM dense on electron microscopic examination, but in the rectum, they are EM lucent. By LM, using histochemical stains, differences are seen in ascending, transverse, and descending cells with mixed magenta and blue-purple staining: in the rectum almost all cells stained blue, indicating highly acid mucus. HID-AB staining showed a large proportion of cells in all four regions staining brown-black, indicating high amounts of sulphomucin.

Pancreas: Pancreatic tissues from immediated autopsy were examined by morphological techniques, histochemistry, immunohistochemistry, and freeze fracture. Pancreatic ducts were maintained using the contractor's organ

B. Autopsy Specimens

Immediate Autopsy: There were five immediate autopsies. The specimens collected are shown below.

<u>Organ</u>	<u>Number of Specimens</u>
Duodenum	5
Colon	5
Bronchus with lung attached	5
Pancreatic duct	5
Pleura	5

Tissues required for pathological examination and assessment of viability were retained by the contractor, but the major portions of the specimens were received at the NIH. Liver samples were quick-frozen in liquid nitrogen and stored at -70°C in the contractor's facility; other samples were shipped in ice-chilled L-15 medium.

Routine Autopsy: The numbers of specimens collected from 78 routine autopsies and other procedures were as listed below:

<u>Organ</u>	<u>Number of Specimens</u>
Bronchus	78
Pleural Fluids	239
Aortas	3

2. Viability Evaluation

Bronchus: Small pieces of nontumorous bronchial epithelium from 10 patients at intermediate autopsy were grown in explant cultures. All of the cases were viable, based upon morphology, cellular outgrowth and metabolic functions tests.

Pancreas: MNU-treated pancreatic duct from immediate autopsy case No. 84-5 was viable when xenotransplanted in nude mice.

Liver: Four liver specimens from immediate autopsies (three from surgery and one from a routine autopsy) were frozen and stored. Liver slices and cells were stored by the contractor and are available upon request for shipment to the NIH.

In the 12-month period, hepatocytes from two livers from immediate autopsy donors were successfully cultured. Viability was determined by the ability to isolate viable cells from a lobe of the liver by a two-stage perfusion method previously described. Cell viability is determined by trypan blue exclusion (positive >70%). Cells isolated and stored from immediate autopsy tissues were approximately 80% viable; those from surgery were frozen without culturing.

explant and cell culture techniques, histocompatibility, immunohistochemistry, and freeze fracture. Routine autopsy and surgically derived tissues were examined to elucidate cellular alterations in pancreatic cancer. Human pancreatic exocrine tumors were collected from the tissue files of the University of Maryland Hospital and the Baltimore VA Medical Center (Loch Raven) and matched by age and sex as controls for marker studies (alpha fetal protein, alcian-blue PAS and keratin) in tissues from the Immediate Autopsy files. Tumor cells have also been isolated from a human exocrine pancreatic tumor and have been passaged and frozen. Samples are in storage at -135°C.

Liver: Samples are collected and portions stored at -70°C. Comparison of methods for the primary culture of human hepatocytes and rat hepatocytes are continuing using different media and substrates, including human liver biomatrix. EM of zero-time samples is used to assess the viability of liver tissue at the time of perfusion. Pieces of liver are also quick-frozen in liquid nitrogen for subsequent use in metabolic studies at the NCI. Results indicate that primary cultures of human liver cells can provide a mechanism for studying chemical metabolism and mutagenesis.

Cytoskeletal Proteins: The cytoskeletal proteins of normal and neoplastic bronchus were studied with immunoperoxidase: Keratin was detected in columnar and basal cells of respiratory epithelium and in duct and serous cells of bronchial glands, actin in apical cytoplasm of ciliated cells, calmodulin in ciliated, basal and serous cells of submucosal glands, and tubulin in cilia. Keratin and actin were detected in all tumors.

Oncogene Proteins: Using specific antibodies to gene products and either PAP or ABC immunohistochemistry, select tissues were assayed for evidence of v-raf, ras^H, or src:

1. Twenty-three of 39 (59%) lung tumors were strongly immunoreactive for v-raf. The positive tissues included 6 of 17 (35%) adenocarcinomas, 16 of 19 (84%) squamous cell carcinomas, and 1 of 1 oat cell carcinoma. Sixteen of the 39 were either negative or gave widely variable results.
2. Two of six (33%) squamous cell carcinomas of the lung were positive for ras^H p21.
3. Four of 16 (15%) lung tumors tested for human endogenous src pp60 were strongly positive. The other cases were either negative (3) or gave weak (7) to moderate (2) responses.

4. Epidemiological Profile Construction and Storage

Abstracting medical records, compiling donor histories, and computerizing these data are essential requirements of this project. In this period, 162 medical records were abstracted for surgery patients (127 for thoracic and 35 for colonic); donor histories were compiled for 56 patients (29 bronchus and 27 colon) via interviews using the standard questionnaire (developed by LHC and the contractor); and in data processing, a total of 145 (118 colonic and 27 thoracic) records (medical and epidemiological) have been coded for computer storage and analysis.

The total number of cases with these data collected from the beginning of the contract (from the seven participating hospitals) are listed below:

	<u>Univ. Hosp.</u>	<u>LRVA</u>	<u>Un. Mem.</u>	<u>St. Agnes</u>
Bronchus	300 (91)	130 (28)	32	60 (25)
Colon	316 (105)	95 (14)	0	0
Total	<u>616 (196)</u>	<u>225 (42)</u>	<u>32</u>	<u>60 (25)</u>

	<u>W. Va.</u>	<u>Sinai</u>	<u>Med. Exam.</u>	<u>Total</u>
Bronchus	0	4	219	745 (144)
Colon	36	0	0	447 (119)
Total	<u>36</u>	<u>4</u>	<u>219</u>	<u>1192 (263)</u>

To date, the number of completions in the efforts to provide epidemiological profiles for donors of tissues delivered in this period are as follows:

	<u>Med. Rec.</u>	<u>Interviewed</u>	<u>Coded</u>	<u>Interview Refused By:</u>			
				<u>Patient.</u>	<u>Doc.</u>	<u>Hos.</u>	<u>Med.</u>
Bronchus	699 (273)	391 (142)	676 (295)	31 (14)	5 (1)	32	219
Colon	381 (145)	321 (144)	367 (176)	22 (6)	5 (2)	0	0
Total	<u>1080 (418)</u>	<u>712 (286)</u>	<u>1043 (471)</u>	<u>53 (20)</u>	<u>10 (3)</u>	<u>32</u>	<u>219</u>

() = 4 year totals for the expired contract.

CONTRACT IN SUPPORT OF THESE PROJECTS: Z01CP05193-06 LHC, Z01CP05427-02 LHC and Z01CP05480-01 LHC

UNIVERSITY OF MARYLAND (N01-CP-31008)

Title: Resource for Human Esophageal Tissues and Cells from Donors with Epidemiological Profiles

Current Annual Level: \$90,105

Man Years: 2.04

Objectives: To provide tissue specimens and cells of human esophagus from epidemiologically defined donors to the Laboratory of Human Carcinogenesis for carcinogenesis studies; to provide fresh, well-characterized, and viable esophageal tissue for primary organ culture at the NIH; to create, characterize, and store monolayer cultures from esophageal tissues for delivery on request to the NIH.

Major Contributions: Sixty-eight specimens were collected and characterized by the contractor. An epidemiological profile of the donors has been provided whenever possible. Morphological, cytochemical, and immunocytochemical characteristics were determined for each tissue collected and will be delivered to the NCI on request. Assays are also being conducted to determine specific biochemical markers (HCG_β, AFP, CEA, etc.) occurring in normal, premalignant, and malignant human esophageal epithelium.

Tissues from the Medical Examiner's (ME) cases are infrequently viable in organ cultures. As a function of time after death, esophagi collected more than 8 hours post-mortem are unlikely to survive in vitro. Monolayer cultures from organ explants are developed according to a modification of the methods of DeBuysscher et al (1984). Explants from "normal" uninvolved immediate autopsies and malignant human esophageal mucosa were cultured in this period. Plating efficiency was 100% for cells from tissues obtained less than 4 hours postmortem. In this period esophageal cells from dog and rabbit were successfully used as controls for culturing, viability, and characterization technology.

The cultures of cells from normal and malignant esophageal mucosa are frozen and thawed as cell stocks in viable condition. Currently, there are approximately 946 vials of frozen stock, including 859 human epithelial cell suspensions (136 normal, 723 malignant), 81 3T3 feeder cells, and 5 sarcoma-180 mouse tumor cells for the production of tumor-conditioned medium, both required for esophageal cell culture.

CONTRACT IN SUPPORT OF THESE PROJECTS: Z01CP05192-06 LHC, Z01CP05193-06 LHC, Z01CP05291-05 LHC, Z01CP05324-04 LHC, Z01CP-05341-04 LHC, Z01CP05424-02 LHC, Z01CP05432-02 LHC, Z01CP05435-02 LHC and Z01CP05480-01 LHC

GEORGETOWN UNIVERSITY (N01-CP-31007)

Title: Collection and Evaluation of Human Tissues and Cells from Donor with an Epidemiological Profile

Current Annual Level: \$74,040

Man Years: 0.96

Objectives: To provide the NCI with (1) a source of human lung and bronchial tissues taken at surgery, (2) pleural fluid from patients with benign and malignant lung disease, (3) human bronchial veolar cells from bronchial lavage of normal smokers and nonsmokers, and (4) completed epidemiological questionnaires for medical and environmental histories.

Major Contributions: In this period, the contractor provided 13 specimens (1 benign, 12 malignant) from selected bronchi and peripheral lung, 9 specimens of pleural fluid for mesothelial cells from patients without malignancies, and 14 specimens of colon from 10 patients with malignancies (mostly adenocarcinomas) and 4 without. Epidemiological (medical and environmental history) profiles were completed for all participating patients and normal volunteers. These records were filed in the contractor's facility for future use by the NCI.

Materials obtained from this contractor were used in ongoing studies in the In Vitro Carcinogenesis and Biochemical Epidemiology Sections of LHC. The human bronchial and peripheral lung tissues are used in ongoing studies of chemical carcinogenesis and human lung cancer. The mesothelial cell cultures from pleural fluids are used to examine in vitro effects of asbestos and other environmental agents that may be involved in the pathogenesis of malignant mesothelioma. The solid specimens (lung and colon) are also tested for carcinogen DNA adducts, ability to repair DNA damage, and genetic polymorphisms.

CONTRACT IN SUPPORT OF THESE PROJECTS: Z01CP05193-06 LHC, Z01CP05291-05 LHC, Z01CP05324-04 LHC, Z01CP05341-04 LHC, Z01CP05432-02 LHC, Z01CP05435-02 LHC and Z01CP05480-01 LHC

VETERANS ADMINISTRATION HOSPITAL (Y01-CP-30255)

Title: Resource for Procurement of Human Tissues from Donors with an Epidemiological Profile

Current Annual Level: \$62,251

Man Years: 1.6

Objectives: This interagency agreement provides (1) specimens of normal, premalignant, and malignant human lung and colon tissues (taken at the time of surgery) for the study of human epithelial responses to carcinogens in cell and organ cultures and as xenotransplants in immunodeficient mice; (2) morphologic and pathologic characterization by light and electron microscopy and histochemistry of normal, premalignant, and malignant epithelium for each tissue; and (3) an epidemiological profile (including preoperative medical and environmental histories) for each donor.

Major Contributions: From lung and colon surgeries, the contractor delivered a total of 136 specimens: 52 lung (25 normal, 27 malignant); 38 colon (20 normal, 18 malignant); 12 bronchus and 32 pleura. All tissues received were characterized by light and electron microscopy. Of the 52 patients who variously underwent pneumonectomy, lobectomy, bilobectomy, colectomy, and local excision, 12 (23%) had squamous cell carcinoma, 6 (12%) had adenocarcinoma of the lung, and 14 (27%) had adenocarcinoma of the colon. Cooperating donors have epidemiological profiles completed and filed in the contractor's facility.

Thus, the contractor continues to obtain acceptable numbers and kinds of tissues from surgical procedures that include pneumonectomy, lobectomy, mediastinal resection, colectomy, gastro-esophagectomy and local excision. In this period 32 malignant lesions were diagnosed in lung (50%), colon (44%) and esophagus (6%). The lung tumors were both squamous cell (65%) and adenocarcinoma (35%), while the colon tumors were all adenocarcinomas. There was one squamous cell carcinoma of the esophagus.

Twenty-five of the 52 patients did not harbor a malignancy, but had a number of other conditions ranging from normal epithelium to granuloma.

CONTRACT IN SUPPORT OF THESE PROJECTS: Z01CP05193-06 LHC, Z01CP05291-05 LHC, Z01CP05324-04 LHC, Z01CP05341-04 LHC, Z01CP05432-02 LHC, Z01CP05435-02 LHC and Z01CP05480-01 LHC

NATIONAL NAVAL MEDICAL CENTER (Y01-CP-30257); WALTER REED ARMY MEDICAL CENTER (Y01-CP-30504)

Title: Procurement of Human Tissues from Donors with an Epidemiological Profile

Current Annual Level: (\$17,364 - deobligated)

Man Years: 1.0

Objectives: (1) To provide pathologically characterized specimens of malignant and noninvolved bronchial, lung and colonic epithelium (obtained at time of surgery for cancer or for benign lesions), with epidemiological profiles of medical and environmental histories for each donor to the NIH for the study of carcinogen activation and deactivation in human tissue and (2) to determine the ability of human tissue to metabolize carcinogens to mutagens.

Major Contributions: Subsequent to staffing problems (resulting in the dismissal of the designated PI for the agreement at the National Naval Medical Center [NNMC]), LHC continued the agreement with the transfer of administrative responsibilities to the Cardiothoracic Surgery Department at Walter Reed. The tissue collection technician was hired in this period for tissue collection from both NNMC and Walter Reed surgeries.

Unfortunately, the new technician suffered a serious illness near the end of the training for the collection duties and had to be separated. However, LHC, Walter Reed and NNMC collaborators continue to be enthusiastic about the revitalization of this project. It is expected that, when the technician is hired, Walter Reed Army Hospital will double the tissue yields (both surgeries average about 75 patients/yr) and stabilize the technical aspects with more experienced management.

CONTRACT IN SUPPORT OF THESE PROJECTS: Z01CP05193-06 LHC, Z01CP05293-05 LHC, Z01CP05324-04 LHC, Z01CP05341-04 LHC, Z01CP05409-03 LHC, Z01CP05410-03 LHC, Z01CP05424-02 LHC, Z01CP05427-02 LHC, Z01CP05431-02 LHC and Z01CP05478-01 LHC

LITTON BIONETICS (N01-CP-51004, replaces N01-CP-15769)

Title: Resource for Xenotransplantation Studies of Carcinogenesis of Human Tissues in Athymic Nude Mice

Current Annual Level: \$317,735

Man Years: 2.70

Objectives: To provide an immunodeficient animal model, the athymic nude mouse, for (1) long-term survival of human tissue xenografts; (2) a continuing resource of athymic nude mice for long-term xenotransplantation, proliferation, and tumorigenicity studies of normal, premalignant, and malignant human tissues; (3) to study the in vivo development of preneoplastic and neoplastic transformation in human tissues induced in vitro and in vivo by selected chemical agents, cellular manipulations, and genetic transfections.

Major Contributions: Human bronchus, pancreatic duct, colon, breast, prostate, and esophagus are maintained for 16 months and beyond as xenografts, as evidenced by viable-appearing epithelium with normal histology, incorporation of labeled precursors into epithelial cells of the grafts, and positive test for human isozymes.

In the breeding stock, 563 Swiss litters contained 3,906 pups (6.9/litter), including 1,842 nu/nu pups. Sixty-three percent of these newborns survived, giving a total of 1,160 or 2.2 surviving nudes/litter. During the year, the contractor maintains a monthly average colony population of 778 mice: 110 breeders, 174 newborns, 48 weanlings for new experiments, and 446 mice in experimental protocols. An average of 8 experimental animals died and 47 were killed monthly.

Nine new experiments were initiated in this period. These included five new experiments (32 ongoing, with 1,098 mice) to study the growth rate and morphology of xenotransplanted HUT 294 cells, a bronchial epithelial cell line, implanted with and without a variety of X-irradiated feeder cells, irradiation of the host and treatment of the host with anti-interferon, antiserum or the implant treated with dimethylnitrosamine, cigarette smoke condensate, nickel (Ni), chromium, or arsenic. Three new experiments with mesothelioma cells (11 ongoing, with 69 mice) were started to study growth potential, morphology and transplantability of xenotransplants by different routes and with or without irradiation, and to determine the response of cells to Amosite asbestos. There was one new experiment added to 24 ongoing experiments (263 mice) to study (a) the morphology of xenotransplanted normal human esophageal (HE) tissue after treatment with N-methyl-N-nitro-N-nitrosoguanidine (MNNG), dimethylbenzanthracene, or dimethylsulfoxide; (b) growth of HE cells in antilymphocyte serum (ALS)-treated or X-irradiated mice; and (c) growth of HE carcinoma cells in normal nude mice; experiments to investigate xenographic characteristics of human pancreatic, and hepatic cells in nude mice. Other ongoing studies include experiments on xenographic growth and morphology of human pancreas and bladder tissues with and without MNNG

and morphology of human pancreas and bladder tissues with and without MNNG treatment or exposure to SV40 + Ni_3S_2 before xenotransplantation into ALS-treated nude mice.

The contractor maintained a 6 month average of 83 ongoing experiments requiring 747 mice, 538 of which survived the period. Twenty experiments were completed during the year and 68 remain ongoing.

Malignant transformation from tissue treated chemically in vitro continues to elude observation in xenografts. Carcinogen induced in vitro induced abnormalities of epithelial cells and tissues do not maintain transformation for sufficiently extended periods as xenografts. Squamous metaplasia also occurs in grafts given carcinogens in vivo but have not become malignant.

The successful xenotransplantation of oncogene-transfected human bronchial epithelial (HBE) cell lines continues to produce tumors. One hundred percent of the nu/nu mice given subcutaneous injections of HBE cells transformed in vitro by transfection with the Harvey ras developed progressively growing nodules. Mice given inoculums of soft agar selected transformed cells develop tumors earlier, but those without soft agar selection developed tumors during extended observations. Resulting tumors have maintained human characteristics (i.e., isozyme positive) through as many as five passages and continue to transplant.

CONTRACT IN SUPPORT OF THESE PROJECTS: Z01CP05326-04 LHC, Z01CP05328-04 LHC, Z01CP05434-02 LHC

BRATON BIOTECH, INC. (N01-CP-51086)

Title: Immunologic Studies of High Risk Groups

Current Annual Level: \$376,065

Man Years: 0.75

Objectives: The contractor provides the following capabilities: HLA typing, Analysis of cell-surface components by flow microfluorometry, development of hetero and human monoclonal antibodies, and implementation of analysis of immune function. In addition, this contractor provides the resource for the development of monoclonal antibody to carcinogen DNA adducts.

Major Contributions: Analysis of cell-surface antigens on lymphocytes from AIDS patients or at-risk populations have been studied during the past year. These studies are in collaboration with the Environmental Epidemiology Branch, NCI. Cell-surface antigen profiles have also been determined on freshly explanted tumor cells from patients with small cell lung carcinoma as well as small cell lung carcinoma cell culture. HLA typing has been performed on the AIDS patients and at-risk populations. HLA typing is being performed on families where the debrisquin phenotype has been determined. A variety of cell lines infected with HTLV-I, II, or III are being carried in the appropriate P3 facilities. These cell lines are being used to study the immunobiology of HTLV-I, II, and III. Mice have been immunized with fecapentaene and monoclonal antibodies developed against this compound deducted to DNA. The results so far suggest that these antibodies are detecting a specific cross-link between the DNA and the fecapentene since the majority of these antibodies react also with psoralen cross-linked to DNA. B cell lines have been established from several patients with HTLV-III infection. These cell lines induced to produce immunoglobulins which have antibody activity to HTLV-III. B cell tumors from two patients with AIDS were explanted and placed in culture. These B cells do not contain the HTLV-III retrovirus. Supernatants from these cultures have been tested and found to have antibody activity against the HTLV-III retrovirus.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05192-06 LHC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Repair of Carcinogen-Induced Damage in Human Cells		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Curtis C. Harris	Chief LHC NCI
Other:	Simon Plummer	Visiting Fellow LHC NCI
COOPERATING UNITS (if any) Department of Physiology, Hershey Medical Center, Hershey, PA (A.E. Pegg), Department of Pathology, University of Maryland School of Medicine, Baltimore, MD (B.F. Trump); Karolinska Institute, Stockholm, Sweden (R.C. Grafstrom)		
LAB/BRANCH Laboratory of Human Carcinogenesis		
SECTION Carcinogen Macromolecular Interaction Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 0.5	PROFESSIONAL: 0.5	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard, unreduced type. Do not exceed the space provided.) Normal adult human tissues and cultured bronchial epithelial cells and fibroblasts exhibit O6-alkylguanine-DNA alkyltransferase activity in vitro by catalyzing the repair of the promutagenic alkylation lesion O6-methylguanine from DNA. Alkyltransferase activity varies in the different human tissues tested in the decreasing order of liver, colon, esophagus, peripheral lung and brain. Various human tissues exhibit 2- to 10-fold higher alkyltransferase activity than corresponding rat tissues. The present results show that different human tissues and cells have a several-fold higher capacity to repair O6-methylguanine in DNA than rat tissues and that the repair process occurs via a mechanism similar to that previously shown in other mammalian cells and <i>E. coli</i> . Formaldehyde inhibits repair of O6-methylguanine and potentiates the mutagenicity of an alkylating agent, N-methyl-N-nitrosourea, in normal human fibroblasts. Because formaldehyde alone also causes mutations in human cells, formaldehyde may cause genotoxicity by a dual mechanism of directly damaging DNA and also inhibiting repair of mutagenic and carcinogenic DNA lesions caused by other chemical and physical carcinogens. In some experimental studies, repeated exposure to alkylating agents has led to an increase in O6-methylguanine-DNA alkyltransferase activity, i.e., an adaptive response. We have shown that human bronchial epithelial cells do not adapt and increase their DNA repair capability. This finding has important implications in carcinogenesis caused by low doses of N-nitrosamines. Membrane damage by carcinogens and tumor promoters can also lead to generation of lipid peroxidation aldehydes and indirect DNA damage. We have recently shown that these aldehydes cause extensive DNA damage and inhibit DNA repair in human bronchial epithelial cells. Studies investigating the activity of O6-methylguanine-DNA alkyltransferase and uracil-DNA glycosylase in the macrophages and peripheral blood lymphocytes of smokers and non-smokers have shown up to 50- and 100-fold differences, respectively, in these activities between individuals of each group. Intra-individual variations were up to sixfold between samples taken at two different time points over a 1-6 month period.		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Curtis C. Harris	Chief	LHC	NCI
Simon Plummer	Visiting Fellow	LHC	NCI

Objectives:

To understand the mechanism of repair of DNA damage by environmental agents in human epithelial tissues and cells and to investigate the genotoxicity of formaldehyde and other tobacco smoke-related aldehydes.

Methods Employed:

Culture of human epithelial and fibroblastic cells; alkaline elution techniques for detection of DNA single strand breaks (SSB) and DNA protein cross-links (DPC); BND cellulose chromatography for measurement of repair replication; ^3H -thymidine incorporation in the presence of hydroxyurea for measurement of unscheduled DNA synthesis; isolation of cellular macromolecules; high pressure liquid chromatography. O^6 -Alkylguanine-DNA alkyltransferase activity found in extracts from a variety of human tissues was characterized and quantitated in three ways: (1) measuring the specific loss of labeled O^6 -methylguanine (O^6 -MeGua) from a ^3H -methylated DNA substrate, (2) measuring the production of protein containing S- ^3H -methylcysteine during the reaction with this DNA substrate and (3) measuring the formation of ^3H -guanine in DNA when the extracts were incubated with a synthetic DNA substrate containing O^6 -MeGua labeled in the 8-position, (4) measuring the removal of ^3H -deoxyuracil monophosphate from DNA when ^3H -dUMP-DNA was incubated with cell supernatant fractions. O^6 -Alkylguanine-DNA alkyltransferase and uracil-DNA glycosylase were measured in the supernatant fraction of homogenates of peripheral blood lymphocytes and macrophages (from bronchial lavage) obtained from smokers and nonsmokers.

Major Findings:

We have investigated alkyltransferase activity in various human tissues and compared it with the corresponding rat tissues. The alkyltransferase activities of cultured normal human bronchial epithelial cells and fibroblasts were also compared. Extracts of human colon, esophagus, and lung had lower activities than those previously found in human liver samples that showed somewhat higher activities than human brain. When compared with the corresponding rat tissue, human tissue samples contained 2- to 10-fold higher levels of alkyltransferase activity. The activity of alkyltransferase and uracil-DNA glycosylase was measured in the macrophages (obtained from bronchial lavage) and peripheral blood lymphocytes in 13 smokers and 16 nonsmokers. Interindividual and intraindividual variations were up to 100-fold and 6-fold, respectively.

Formaldehyde (HCHO) is a common environmental pollutant found in tobacco smoke and a metabolite of demethylation reactions of drugs and carcinogenic N-nitrosamines. It is also a respiratory carcinogen in rats and a potential carcinogenic hazard in humans. Therefore, we have initiated a systematic study of the

genotoxicity of HCHO in cultured human cells. The alkaline elution technique was used to study repair of DNA damage caused by HCHO in human bronchial epithelial cells and fibroblasts, skin fibroblasts, and DNA excision repair-deficient skin fibroblasts from donors with xeroderma pigmentosum (XP). Exposure of cells to HCHO resulted in DNA-protein cross-links (DPC) and DNA single strand breaks (SSB) in all cell types. DPC were induced at similar levels and were also removed by all cell types, including the XP cells. By excision repair of HCHO-induced DNA damage, normal cells generated SSB that were also readily repaired. HCHO was only moderately cytotoxic to normal bronchial epithelial cells and fibroblasts at concentrations that induced substantial DNA damage. HCHO enhanced the cytotoxicity of both ionizing radiation and N-methyl-N-nitrosourea in both cell types. The results indicate that most DPC caused by HCHO can be removed without the involvement of DNA excision repair. Furthermore, HCHO also directly causes DNA SSB as well as SSB generated indirectly during UV-type excision repair. These studies indicate the complexity of the HCHO-induced DNA damage and its repair and that HCHO may enhance the cytotoxicity of chemical and physical carcinogens in human cells.

Since HCHO is formed in equimolar quantities with methylcarbonium ions during the metabolic activation of N-nitrosodimethylamine, we have recently examined the effects of HCHO on the repair of the promutagenic lesion O⁶-MeGua formed following N-nitrosodimethylamine metabolism. HCHO decreases O⁶-alkyltransferase activity, inhibits the removal of O⁶-MeGua, and in low concentrations, synergistically potentiates the cytotoxicity and mutagenicity of N-methyl-N-nitrosourea. In high doses (100 or 130 μ M), HCHO is detectably mutagenic itself. Therefore, exposure to HCHO may lead to the dual genotoxic mechanism of both directly damaging DNA and inhibiting repair of mutagenic and carcinogenic lesions caused by alkylating agents and physical carcinogens.

Normal human bronchial epithelial cells cultured in serum-free medium were exposed to low doses of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) to examine whether increased cellular resistance and increased activity of the DNA repair enzyme O⁶-methylguanine-DNA methyltransferase could be induced. After treatment with single doses of MNNG a dose-dependent decrease in O⁶-methylguanine-DNA methyltransferase activity was observed, as expected, for this unique repair system. The activity recovered to the starting level in about 24 hours when a dose that consumed approximately 65% of the enzyme activity (0.2 μ g/ml) was given, but did not exceed the activity in the untreated control. Furthermore, treatment every 6 hours for 4 to 5 days with nontoxic concentrations of MNNG (0.04-0.12 μ g/ml) did not increase O⁶-methylguanine-DNA methyltransferase activity. Neither was cell survival following a range of challenge doses significantly increased. Our data suggest that human bronchial epithelial cells do not adapt to MNNG.

Lipid peroxidation aldehydes of the 4-hydroxy- α,β -unsaturated type as well as the tobacco-smoke related α,β -unsaturated aldehyde, acrolein, were highly cytotoxic and reduced intracellular thiols in cultured human bronchial fibroblasts after treatment with micromolar concentrations. In comparison, formaldehyde and acetaldehyde were less toxic and 100- to 300-fold higher doses were required to affect survival or thiol levels. The unsaturated aldehydes also inhibited the DNA repair enzyme O⁶-methylguanine-DNA methyltransferase known to have a cysteine residue in its active site, but had no effect on the activity of uracil-DNA glycosylase. Our results indicate that reactive aldehydes of exogenous or endogenous origin have direct cytotoxic effects and may also make cells more

susceptible to other toxic chemicals due to a reduction in cellular defense mechanisms, e.g., DNA repair and detoxification by systems requiring glutathione.

Publications:

Grafstrom, R. C., Curren, R. D., Yang, L. L. and Harris, C. C.: Aldehyde-induced inhibition of DNA repair and N-nitroso compound-induced mutagenesis in human cells. In Krokan, H. and Myrnes, B. (Eds.): Fourth International Conference on Environmental Mutagens. New York, A. R. Liss. (In Press)

Grafstrom, R. C., Pegg, A. E., Harris, C. C., Sundquist, K., Krokan, H.: O⁶-methylguanine-DNA methyltransferase activity and aldehyde-induced inhibition of O⁶-methylguanine repair in human lung cells. In Myrnes, B. and Krokan, H., (Eds.): Repair of DNA Lesions Introduced by N-nitroso-compounds. England, Oxford University Press. (In Press)

Grafstrom, R., Willey, J., Sundquist, K. and Harris, C. C.: Pathobiological effects of tobacco-related aldehydes in cultured human bronchial epithelial cells. In Hoffmann, D. and Harris, C. C. (Eds.): Banbury Report 23. Mechanisms of Tobacco Carcinogenesis. Cold Spring Harbor, Cold Spring Harbor Laboratory. (In Press)

Grafstrom, R. C., Willey, J. C., Sundquist, K. and Harris, C. C.: Toxicity of tobacco-related aldehydes in cultured human bronchial epithelial cells. U.S. Air Force Technical Report 84-002: 255-265, 1985.

Krokan, H., Grafstrom, R. C., Sundquist, K. and Harris, C. C.: Cytotoxicity, thiol depletion, and inhibition of O⁶-methylguanine-DNA methyltransferase by various aldehydes in cultured human bronchial cells. Carcinogenesis 6: 1755-1759, 1985.

Krokan, H., Haugen, A., Giercksky, K.-E., Nilson, I. W., Yoakum, G. H., Harris, C. C. and Myrnes, B.: O⁶-methylguanine-DNA methyltransferase activity in human tissues and the possible suppressive effect of some activated ras-oncogenes in cultured cells. In Krokan, H. (Ed.): Repair of DNA Lesions Introduced by N-Nitroso-compounds. England, Oxford University Press. (In Press).

Krokan, H., Lechner, J., Krokan, R. H. and Harris, C. C.: Normal human bronchial epithelial cells do not show an adaptive response after treatment with N-methyl-N'-nitrosoguanidine. Mutation Res. 146: 205-209, 1985.

Saladino, A. J., Willey, J. C., Lechner, J. F., Grafstrom, R. C. and Harris, C. C.: Effects of formaldehyde, acetaldehyde, benzoyl peroxide, and hydrogen peroxide on cultured human bronchial epithelial cells in vitro. Cancer Res. 45: 2522-2526, 1985.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05193-06 LHC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Growth and Differentiation of Normal Human Epithelial Cells and Carcinoma Cells		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: Susan P. Banks-Schlegel Sr. Staff Fellow LHC NCI Others: Curtis C. Harris Chief LHC NCI		
COOPERATING UNITS (if any) University of Alabama, Birmingham, AL (K. Sexton); University of Maryland (B. Trump).		
LAB/BRANCH Laboratory of Human Carcinogenesis		
SECTION In Vitro Carcinogenesis Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 2.2	PROFESSIONAL: 1.2	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Squamous cell carcinomas have recently been shown to contain increased numbers of epidermal growth factor (EGF) receptors. Since EGF has an important role in epithelial growth and differentiation, it is possible that modulation of its receptor may have an important role in neoplasia. In an attempt to further explore the relationship of EGF receptor expression to malignant transformation, we examined 14 squamous cell carcinoma cell lines of the esophagus for the number and affinity of EGF receptors. Seven cell lines were newly isolated by this laboratory and recently characterized. The seven additional cell lines were obtained from Japan (4 cell lines) and South Africa (3 cell lines). Surprisingly, we found that esophageal carcinomas contained lowered quantities of surface EGF receptors (2- to 100-fold) and that the affinity of the EGF receptor was increased (6- to 100-fold) when compared to normal esophageal epithelial cells. Moreover, the biologic response of esophageal carcinoma cells to EGF differed markedly from that of other squamous cell tumor cells exhibiting elevated numbers of receptors, such as A431 and SCC-15. Human esophageal carcinoma cells were maximally stimulated by the addition of 5 ng/ml of EGF, similar to normal esophageal keratinocytes, but in contrast to normal cells were not inhibited by the higher concentrations tested (up to 40 ng/ml). On the other hand, addition of any EGF to the medium (beyond that normally present in serum) was found to dramatically inhibit the growth of A431 and SCC-15 cells. Our findings indicate that squamous cell neoplasia is not dependent upon increased numbers of cell surface EGF receptors, that EGF receptor number may have a determinant role in EGF cell toxicity, and that the stimulatory response of cells to EGF may reflect a complex function of EGF receptor number, affinity, and occupancy.		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on This Project:

Susan P. Banks-Schlegel	Sr. Staff Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

Because EGF is an important regulator of normal squamous epithelial growth and differentiation and because EGF receptor number has been shown to be elevated in all squamous cell carcinoma cells examined thus far, thus suggesting its possible role in malignant growth, we sought to investigate whether EGF might have differential effects on normal and transformed esophageal epithelial cells and whether the EGF receptor was altered in the transformed cells. Using cell lines isolated by our laboratory, as well as those obtained from others, we found two important and interesting differences in the EGF receptors of esophageal carcinoma cells which don't correlate with previous findings for squamous cell carcinomas from other anatomic sites; esophageal cells have fewer receptors with increased ligand affinity. Moreover, these esophageal tumor cells with fewer receptors are much more tolerant of increased levels of EGF compared to squamous tumors with high levels of EGF receptor.

Methods Employed:

Human esophageal epithelial cells and esophageal carcinoma cell lines (HCE Series) were isolated and grown by procedures developed in this laboratory. Additional cell lines were obtained from Japan and South Africa. Duplicate cultures were assessed for their ability to bind ^{125}I -EGF using standard assays. The kinetics and concentration dependence of EGF binding was assessed and Scatchard analysis of the steady state binding data performed. In addition, normal and transformed cells were examined for differential effects in their biologic response to EGF. Cells (clonal density) were grown in the presence of increasing concentrations of EGF. When any of the experimental dishes reached 80% confluence, all of the experimental dishes were fixed and stained with Rhodanile blue, specific for epithelial cells.

Major Findings:

The role of growth factors and their receptors in transformation is of increasing interest. Because of the importance of EGF to normal squamous epithelial growth and differentiation and because the presence of elevated levels of EGF receptors in squamous cell carcinomas derived from a variety of sources (skin, head and neck, lung, and cervix) suggested its possible role in malignant growth of these cells, we examined 14 human esophageal carcinoma cell lines for alterations in the biologic response to EGF or the EGF receptor level compared to normal esophageal keratinocytes.

We have found that transformed esophageal cells have an altered growth response to EGF and have fewer receptors with an increased affinity of binding for EGF. These findings contrast with those of other laboratories demonstrating

first, increased levels of EGF receptor in all squamous cell carcinomas (from skin, head and neck, lung, and cervix) examined thus far, both in cell lines and biopsy specimens, and second, growth inhibition of squamous epithelial cell lines by concentrations of EGF mitogenic for normal keratinocytes. Our results suggest an interesting correlation between EGF receptor number and the biologic behavior of the cells; namely that cells with fewer receptors are more tolerant of increased levels of EGF, whereas cells with increased numbers of receptors are inhibited by concentrations of EGF mitogenic for normal cells. Moreover, our studies show that the presence of high levels of EGF receptors is not always a hallmark of squamous cell malignancies and that squamous tumors can and do arise without an elevation in EGF receptor levels or markedly altered biologic responses to EGF. In this regard, most of the esophageal carcinoma lines examined in this study, which were derived from human tumors, were tumorigenic when injected subcutaneously into athymic nude mice, even HCE-3 which possessed 100-fold fewer receptors than normal esophageal epithelial cells and was maximally stimulated to grow by concentrations of EGF similar to that found for normal keratinocytes.

An apparent loss of EGF-binding sites on the surfaces of transformed cells has been noted previously and one study demonstrated a correlation between decreased EGF receptor levels and the stage of neoplastic progression. In some, but not all cases, the decreased level of surface EGF receptor correlated with the autocrine production of transforming growth factors (TGF). Two findings indicate that the decrease in EGF receptors on esophageal carcinoma cells is not due to autocrine TGF production. First, cells producing TGF usually do not bind EGF and are unresponsive to EGF in the medium. The fact that the esophageal carcinoma cells can bind EGF and are maximally stimulated to grow by concentrations of EGF required to optimally grow normal cells makes this possibility seem unlikely. Second, incubation of normal cells or A431 cells with conditioned medium from the carcinoma cells did not reduce their binding of ^{125}I -EGF.

In the present study, we found no simple relationship between receptor number (capacity), affinity, occupancy, and the growth-stimulatory response to EGF. Maximal growth stimulation of normal and malignant cells occurred at sub-saturating concentrations of EGF (when 10% of the cell surface receptors were occupied in the case of normal cells and when 60 to 80% of the cell surface receptors were occupied in the case of the esophageal carcinoma cells). Obviously this means that different numbers of receptors are being occupied on normal and transformed cells in order to achieve a biological response and that it is not easy to reconcile cell stimulation as the direct biochemical consequence of cell receptor activity.

Publications:

Banks-Schlegel, S.P.: Isolation, cultivation, and characterization of normal human esophageal epithelial cells. J. Tissue Cult. Methods 9: 95-105, 1985.

Banks-Schlegel, S.P. and Quintero, J.: Growth and differentiation of human esophageal carcinoma cell lines. Cancer Res. 46: 250-258, 1986.

Banks-Schlegel, S.P. and Quintero, J.: Human esophageal carcinoma cells have fewer, but higher affinity epidermal growth factor receptors. J. Biol. Chem. 261: 4359-4362, 1986.

Banks-Schlegel, S.P. and Rhim, J.: Keratin expression by both chemically and virally transformed human epidermal keratinocytes during the process of neoplastic conversion. Carcinogenesis 7: 153-157, 1986.

Burg-Kurland, C. L., Purnell, D. M., Combs, J. W., Hillman, E. A., Harris, C. C. and Trump, B. F.: Immunocytochemical evaluation of human esophageal neoplasms and preneoplastic lesions for beta-chorionic gonadotropin, human placental lactogen, alpha-fetoprotein, carcinoembryonic antigen, and nonspecific cross-reacting antigen. Cancer Res. 46: 2936-2943, 1986.

Trump, B. F., McDowell, E. M., Shamsuddin, A. K. M., Phelps, P. C., Purnell, D. M., Wilson, T. S., Banks-Schlegel, S. P., Kurland, C. L. and Harris, C. C.: Preneoplasia and neoplasia of the bronchus, esophagus and colon: The use of markers in determining phenotypes and classification. In Scarpelli, D. and Craighead, J. (Eds.): The Pathologist and the Environment. Baltimore, Williams and Wilkins, 1985, pp. 101-139.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER 201CP05291-05 LHC																												
PERIOD COVERED October 1, 1985 to September 30, 1986																														
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) DNA Adducts in People Exposed to Polycyclic Aromatic Hydrocarbons																														
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 30%;">PI: C. C. Harris</td> <td style="width: 30%;">Chief</td> <td style="width: 20%;">LHC</td> <td style="width: 20%;">NCI</td> </tr> <tr> <td colspan="4" style="padding-top: 10px;">Others:</td> </tr> <tr> <td>D. L. Mann</td> <td>Section Chief</td> <td>LHC</td> <td>NCI</td> </tr> <tr> <td>G. E. Trivers</td> <td>Biologist</td> <td>LHC</td> <td>NCI</td> </tr> <tr> <td>A. Weston</td> <td>Visiting Fellow</td> <td>LHC</td> <td>NCI</td> </tr> <tr> <td>S. Plummer</td> <td>Visiting Fellow</td> <td>LHC</td> <td>NCI</td> </tr> <tr> <td>V. Wilson</td> <td>Sr. Staff Fellow</td> <td>LHC</td> <td>NCI</td> </tr> </table>			PI: C. C. Harris	Chief	LHC	NCI	Others:				D. L. Mann	Section Chief	LHC	NCI	G. E. Trivers	Biologist	LHC	NCI	A. Weston	Visiting Fellow	LHC	NCI	S. Plummer	Visiting Fellow	LHC	NCI	V. Wilson	Sr. Staff Fellow	LHC	NCI
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A. Weston	Visiting Fellow	LHC	NCI																											
S. Plummer	Visiting Fellow	LHC	NCI																											
V. Wilson	Sr. Staff Fellow	LHC	NCI																											
COOPERATING UNITS (if any) Mt. Sinai Sch. of Med., New York, NY (R.R. Boesch); Univ. of MD Sch. of Med., Baltimore, MD (B.F. Trump); Georgetown Univ. Sch. of Med., Washington, D.C. (H. Yeager); Univ. of CA Sch. of Med., Los Angeles, CA (W. Wright)																														
LAB/BRANCH Laboratory of Human Carcinogenesis																														
SECTION Biochemical Epidemiology Section																														
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892																														
TOTAL MAN-YEARS: 2.0	PROFESSIONAL: 2.0	OTHER:																												
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews																														
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Benzo[a]pyrene (BP), a ubiquitous carcinogen found in tobacco smoke, burning fossil fuels and animal and human diets, forms BP diol epoxide (BPDE)-DNA adducts in animals and humans sufficiently exposed. However, human BPDE-DNA adduct levels would most likely be low and beyond the sensitivity of routine radioimmunoassay and chromatographic analysis. Therefore, enzyme-linked immunosorbent assay (ELISA), ultrasensitive enzymatic radioimmunoassay (USERIA), and synchronous fluorescence spectrophotometry (SFS) have been employed to study these markers in subpopulations in humans with cancer who are considered at high risk for cancer, due in part to BP exposure. With these assays, putative BPDE-DNA adducts have been detected and quantitated in DNA isolated from peripheral blood lymphocytes (PBLs) of several asphalt workers (roofers), foundry workers (coke oven), and head and neck, and lung cancer patients; in DNA from lung tissue, bronchial washings, and areolar macrophages of some lung cancer patients; and smoking and nonsmoking, occupationally unexposed, normal volunteers. Antibodies to BPDE-DNA adducts were also found in sera from highly exposed coke oven workers. These data support the conclusion that the activation of BP to its ultimate carcinogen and the subsequent adduction of the metabolite to DNA occurs in humans. Preliminary evidence indicates that other polycyclic aromatic hydrocarbons (i.e., chrysene and benzo[a]anthracene-DNA adducts) may also induce immune responses in humans.</p>																														

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Curtis Harris	Chief	LHC	NCI
Dean L. Mann	Section Chief	LHC	NCI
Glennwood E. Trivers	Biologist	LHC	NCI
Ainsley Weston	Visiting Fellow	LHC	NCI
Simon Plummer	Visiting Fellow	LHC	NCI
Vincent Wilson	Sr. Staff Fellow	LHC	NCI

Objectives:

Using rabbit anti-benzo[a]pyrene diol epoxide (BPDE) antibodies and the most sensitive immunoassays available, BPDE-DNA adducts and anti-BPDE-DNA antibodies will be determined in human high risk subpopulations. The results will contribute to increased understanding of carcinogen activation and mechanisms of carcinogenesis in humans.

Methods Employed:

Sedimented cell pellets of alveolar macrophages and bronchial washings and 25 to 40 ml of peripheral blood was obtained from 30 male and female normal volunteers and 42 coke oven workers who were smokers, ex-smokers and nonsmokers. The blood samples were centrifuged at 100 x g for 15 minutes and the "buffy coat" was homogenized in 5 volumes of HKM: 0.25 M sucrose buffer (0.05 M HEPES, pH 7.3; 0.24 M (KCl; 0.05 M MgCl₂). Each pellet (including bronchial and macrophage) was suspended in HKM-sucrose buffer containing 0.5% triton 100 and centrifuged for 10 minutes at 4°C. The pellet was suspended in HKM-sucrose buffer and recentrifuged. The final pellet was resuspended in 5 ml HKM-sucrose buffer containing 1% SDS and 1 M NaCl. An equal volume of chloroform-isoamyl alcohol (24:1, v/v) was added, and the mixture was vigorously agitated for at least 20 minutes followed by centrifugation at 10,000 x g for 10 minutes. The aqueous epiphase was removed by winding onto a glass rod. Residual ethanol was removed by nitrogen and DNA dissolved in water. Purity and quantitation of DNA were determined by absorbance at 260 nm and 280 nm using a Beckman DU8 spectrophotometer and a fluorometer. The final volume of DNA solution was adjusted up to 1 mg DNA/ml in water and the solution was sonicated and stored at -70° until tested. DNA was similarly isolated from lung tissue. DNA from foundry workers was received in purified form. It was also sonicated and stored frozen at -70°C until tested.

Competitive enzyme immunoassays, USERIA and ELISA, were performed on test DNA using rabbit anti-BPDE-DNA antibody. Polyvinyl u-bottom 96 well microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with unmodified (control) DNA and BPDE-modified DNA (0.2 ng/well for USERIA and 1 ng/well for ELISA). Standard competitive inhibition curves were obtained by mixing serial dilutions of known standard BPDE-DNA with rabbit antisera. Percentage inhibition of the test samples was determined from the standard curves. All tests and assays were done in triplicate, and the standard deviation was less than 10%.

Major Findings:

Coke oven workers receive one of the highest levels of occupational exposure to carcinogenic polycyclic aromatic hydrocarbons, including benzo[a]pyrene (BP) and are at high risk for lung cancer. Since BP is enzymatically activated to 7 β , 8 α -dihydroxy-(9 α , 10 α)-epoxy-7,8,9,10-tetrahydro BP (BPDE) which forms adducts with DNA, detection of these adducts was studied in DNA from peripheral blood lymphocytes by enzyme radioimmunoassay and synchronous fluorescence spectrophotometry. Approximately two-thirds of a group of 42 workers from an older company had detectable levels of BPDE-DNA adducts. Antibodies to the DNA adducts were also found in the sera of 27% of these workers. In a group of 38 workers from a younger, more modern company, 34% had detectable BPDE DNA adducts and 32% had anti-BPDE-DNA serum antibodies in blood samples taken on the job. After a 4 week vacation, all the positive cases retested (70%) for adducts were detectable, but with lower levels. There was also essentially the same number of cases with detectable antibodies, but only 33% of the original cases repeated and at the same levels. To date, 43 of 128 (34%) highly exposed coke oven workers and roofers have had detectable adducts, with mean levels per group 0.2 to 7.1 femtomoles BPDE/ μ g of DNA. Smokers among these positive workers have generally had higher frequencies of positive cases and higher levels of detectability. However, only 6% of 81 occupationally unexposed normal volunteers (32 smokers; 49 nonsmokers) had detectable adducts with levels from 0.17 to 0.94 femtomoles BPDE/ μ g DNA. Adduct levels were higher among smoking than in nonsmoking coke oven workers. There were no smoking related differences in adduct levels of normal volunteers.

Publications:

Gabrielson, E. and Harris, C.C.: Carcinogenic research using culture human tissues and cells. Eur. J. Oncol. Cancer Res. 110: 1-10, 1985.

Harris, C. C.: Future directions for use of carcinogen-DNA adducts as internal dosimeters to monitor human exposure to mutagens and carcinogens. Environ. Health Perspect. 62: 185-191, 1985.

Harris, C. C., LaVeck, G., Groopman, J., Wilson, V. and Mann, D. L.: Measurement of aflatoxin B₁, its metabolites and DNA adducts by synchronous fluorescence spectrophotometry. Cancer Res. (In Press)

Harris, C. C., Vahakangas, K., Autrup, H., Autrup, H., Trivers, G. E., Shamsuddin, A. K. M., Trump, B. F., Boman, B. M., and Mann, D. L.: Biochemical and molecular epidemiology of human cancer risk. In Scarpelli, D., Craighead, J. and Kaufman, N. (Eds.): The Pathologist and the Environment. Baltimore, Williams and Wilkins, 1985, pp. 140-167.

Harris, C. C., Vanakangas, K., Newman, M., Trivers, G. E., Mann, D. L. and Wright, W.: Detection of benzo[a]pyrene diol epoxide-DNA adducts in peripheral blood lymphocytes and antibodies to the adducts in sera from coke oven workers. Proc. Natl. Acad. Sci. USA 82: 6672-6676, 1985.

Haugan, A., Becher, G., Benestad, C., Vahakangas, K., Trivers, G. E., Newman, M. J., Harris, C. C.: Determination of polycyclic aromatic hydrocarbons (PAH) in the urine, benzo[a]-pyrene diol epoxide-DNA adducts in lymphocyte DNA and antibodies to the adducts in sera from coke oven workers exposed to measured amounts of PAH in the work atmosphere. Cancer Res. (In Press)

Vahakangas, K., Trivers, G. E., Rowe, M., and Harris, C. C.: Benzo[a]pyrene diol epoxide-DNA adducts detected by synchronous fluorescence spectrophotometry. Environ. Health Perspect. 62: 101-104, 1985.

Weston, A., Trivers, G. E., Vahakangas, K., Newman, M., Mann, D. and Harris, C. C.: Detection of carcinogen-DNA adducts in human cells and antibodies to these adducts in human sera. Prog. Exp. Tumor Res. (In Press)

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05293-05 LHC																												
PERIOD COVERED October 1, 1985 to September 30, 1986																														
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Oncogene Transfection of Human Cells																														
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 30%;">PI: George H. Yoakum</td> <td style="width: 30%;">Senior Staff Fellow</td> <td style="width: 20%;">LHC</td> <td style="width: 20%;">NCI</td> </tr> <tr><td colspan="4"> </td></tr> <tr> <td>Others: John F. Lechner</td> <td>Section Chief</td> <td>LHC</td> <td>NCI</td> </tr> <tr> <td>Ainsley Weston</td> <td>Visiting Fellow</td> <td>LHC</td> <td>NCI</td> </tr> <tr> <td>James Willey</td> <td>Biotech Training Fellow</td> <td>LHC</td> <td>NCI</td> </tr> <tr> <td>Paul Amstad</td> <td>Visiting Fellow</td> <td>LHC</td> <td>NCI</td> </tr> <tr> <td>Curtis C. Harris</td> <td>Chief</td> <td>LHC</td> <td>NCI</td> </tr> </table>			PI: George H. Yoakum	Senior Staff Fellow	LHC	NCI					Others: John F. Lechner	Section Chief	LHC	NCI	Ainsley Weston	Visiting Fellow	LHC	NCI	James Willey	Biotech Training Fellow	LHC	NCI	Paul Amstad	Visiting Fellow	LHC	NCI	Curtis C. Harris	Chief	LHC	NCI
PI: George H. Yoakum	Senior Staff Fellow	LHC	NCI																											
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Curtis C. Harris	Chief	LHC	NCI																											
COOPERATING UNITS (if any)																														
LAB/BRANCH Laboratory of Human Carcinogenesis																														
SECTION Carcinogen Macromolecular Interaction Section																														
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892																														
TOTAL MAN-YEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 1.0																												
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews																														
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> The hypothesis that transformation of human bronchial epithelial cells (NHBE) following transfection with a v-Ha-ras containing plasmid includes an activity that interferes with the maintenance of normal chromosome was tested. The "karyotypic instability" hypothesis was examined by spreading chromosomes of mitotic cells after protoplast fusion transfection of NHBE cells from several donors with a plasmid (H1) that carries v-Ha-ras. The effects of transient expression of transfected sequences on the population were tested immediately after transfection and one passage later. The results were scored as total chromosomal abnormalities and the observations from four experiments summarized as follows: 1) v-Ha-ras transfected NHBE cells, 45/268 mitosis (16.8%); 2) pBR322 transfected NHBE cells, 8/200 mitosis (4.0%); 3) protoplast fusion no plasmid treated NHBE cells (7.0%); 4) untreated NHBE cells, 17/200 mitosis (8.5%). Thus, a significant increase in chromosomal abnormalities occurs in NHBE cell populations transfected by v-Ha-ras. This effect was observed 24 to 72 hours after introduction of the Ha-ras oncogene, during the period preceding the selection of transformed populations of NHBE cells by challenge with growth conditions that stimulate terminal differentiation of NHBE cells (i.e., 2-4% serum added to serum free LHC-medium). This increase in karyotypic instability is potentially important to the mechanism of v-Ha-ras transformation of NHBE cells and is consistent with the observed requirement for secondary rare events for survival of senescence, establishment of indefinite culture life span, and tumorigenicity of transformed NHBE cells. </p>																														

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

George H. Yoakum	Senior Staff Fellow	LHC	NCI
John F. Lechner	Section Chief	LHC	NCI
Ainsley Weston	Visiting Fellow	LHC	NCI
James Willey	Biotech Training Fellow	LHC	NCI
Paul Amstad	Visiting Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

Study the mechanism of oncogene transformation of normal human bronchial epithelial cells (NHBE) by protoplast fusion transfection. This method permits assessment of both the effects of acute expression and isolation of oncogenes transformed NHBE cells.

Methods Employed:

Protoplast fusion transfection; cell culture.

Major Findings:

Protoplast fusion transfection of NHBE cells with v-Ha-ras was followed by colchamid treatment to observe the effects of v-Ha-ras expression on chromosome stability by direct scoring of mitoses 24, 48, and 72 hours after the transfection. Analysis of the effects on v-Ha-ras expression on NHBE cells required evaluation of approximately 1500 metaphases including the following control cells: i) untreated NHBE cells, ii) protoplast fusion with HB101 containing no plasmid; and iii) NHBE cells transfected with pBR322 carried in HB101. These v-Ha-ras transfected NHBE cells had at least twofold more chromosomal aberrations including hyperdiploid metaphases than the control groups. This finding is of significance to the mechanism of ras-mediated transformation of human cells, suggesting the direct role of ras gene products in the maintenance of stable genomic structure.

The protoplast fusion method was used to transform NHBE by transfection with the pBR322/Ha-MSV plasmid carrying the v-Ha-ras gene (H1). The rescue of episomal forms of v-Ha-ras DNA from the transformed human cell line (TBE-1) isolated by transfection of TBE-1 DNA preparations into *E. coli* followed by selection for Ap^R infers that the transfected plasmid recombined with naturally occurring episomal elements to form a "shuttle-plasmid" capable of replication in human cells and in *E. coli*. The presence of v-Ha-ras on approximately 80% and human Alu-sequences on 100% of the plasmids recovered in this fashion is consistent with the properties required for stable maintenance of transfected DNA in human cells when these genetic elements are covalently linked to appropriate vectors for transfection analysis.

Characterization of v-Ha-ras transfected NHBE cells, including restriction mapping of integration loci, episomal Ha-ras-DNA, selection of tumorigenic and nontumorigenic clones, and determination of conditions required for expression of tumorigenic phenotypes, will provide information to elucidate the mechanisms of ras-mediated carcinogenesis in an important progenitor cell of human lung cancer.

A 7.3 Kbp polymorphic Bam HI fragment containing a copy of v-Ha-ras is present in all of the TBE-series, and the integrated structure of v-Ha-ras has changed as the cells progressed to a more malignant phenotype. In addition, this change in structure of integrated v-Ha-ras sequences may be relevant to the transformed phenotype and the increasing tumorigenicity of TBE-cells (the 5.8 Kbp band).

Publications:

Masui, T., Lechner, J. F., Yoakum, G. H., Willey, J. C. and Harris, C. C.: Growth and differentiation of normal and transformed human bronchial epithelial cells. J. Cell. Physiol. Suppl. (In Press)

Masui, T., Yoakum, G. H., Lechner, J. F., Willey, J. C., Amstad, P., Trump, B. F. and Harris, C. C.: In vitro carcinogenesis studies of human bronchial epithelial cells. In Hoffmann, D. and Harris, C. C. (Eds.): Bantury Report 23. Mechanisms of Tobacco Carcinogenesis. Cold Spring Harbor, Cold Spring Harbor Laboratory. (In Press)

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05324-04 LHC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Human Lung Carcinoma/Bronchial Epithelial Cell Hybrid Genetics		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	John F. Lechner	Section Chief LHC NCI
Others:	Curtis C. Harris	Chief LHC NCI
COOPERATING UNITS (if any) University of California at Irvine, Irvine, CA (E. Stanbridge)		
LAB/BRANCH Laboratory of Human Carcinogenesis		
SECTION In Vitro Carcinogenesis Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 0.5	PROFESSIONAL: 0.2	OTHER: 0.3
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Genetic changes related to carcinogenesis are being studied using hybrids of human lung carcinoma cells with normal human bronchial epithelial cells. Initial studies suggest that a limited population doubling potential (mortality) is the dominant genetic trait in hybrid cells. Other hybrid cell lines have been isolated and are being characterized for doubling potential, karyotype and tumorigenicity in athymic nude mice. The effects of individual chromosomes are being assessed by fusion with minicells containing single marked chromosomes.		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

John F. Lechner	Section Chief	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

Somatic cell genetics of mortality, tumorigenicity and other aspects of transformation will be studied using hybrids of human lung carcinoma cell lines with normal human bronchial epithelial cells.

Methods Employed:

The methods and media for culturing normal human bronchial epithelial cells have been previously developed in this laboratory. Clones of ouabain-resistant HGPRT-lacking cells from established human lung carcinoma cell lines have been derived for the purpose of selecting hybrids.

Cell-cell fusion is done with polyethylene glycol, and hybrids are selected in a media containing HAT (hypoxanthine, aminopterin and thymidine) and ouabain. This selection media is toxic to both the normal parent (ouabain) and the carcinoma parent (HAT).

A mouse/human hybrid cell line A-9 containing one human chromosome 11 with a transposed HGPRT locus has been introduced into the lab. Mini-cells containing only this $\Delta 11$ chromosome are prepared by a series of steps beginning with a 55 hr exposure to colcemid used then, enucleation with cytochalasin B and differential filtration to concentrate the single-chromosome mini-cells.

Methods for measuring the doubling potential of cell lines have been developed utilizing successive passaging of cells and colony size measurement with the Artec image analyzer. Methods for karyotypic analysis of hybrid cell lines are available and tumorigenicity may be assessed by growth in athymic nude mice.

Major Findings:

Initial fusions of HUT 292, a human lung carcinoma cell line, with normal human bronchial epithelial cells and selection of hybrids as described above have resulted in the isolation of several clones. With extended culturing, all of these clones to date have demonstrated a limited doubling potential.

Of the 24 hybrid clones from fusion of H292 DM with cells of a second lung carcinoma line (TBE-1), 11 senesced within 20 population doublings. The 13 clones that have not senesced (and presumably are immortal) are currently being tested for tumorigenicity in nude mice.

None of the 10 hybrid clones from fusion of H292 DM with a third carcinoma line (A1146) have senesced. These clones are also currently being examined for tumorigenicity.

Mini-cells have been produced and fusions with carcinoma lines are underway.

Publications:

None

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05325-04 LHC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) DNA Cytosine Methylation and Cellular Physiology		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: Vincent L. Wilson Sr. Staff Fellow LHC NCI Others: Curtis C. Harris Chief LHC NCI		
COOPERATING UNITS (if any) Clinical Hematology Branch, NHLBI, NIH, Bethesda, MD (R.J. Ley); Gerontology Research Center, NIA, Baltimore, MD (R.G. Cutler); Lab. Environmental Carcinogenesis, Copenhagen, Denmark (H. Autrup).		
LAB/BRANCH Laboratory of Human Carcinogenesis		
SECTION Biochemical Epidemiology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 1.7	PROFESSIONAL: 0.7	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.) <p>The role DNA methylation plays in the promoter regions of two separate gene systems has been clarified. Demethylation of at least one Hpa II restriction site upstream from the structural coding sequences of human gamma globin or the hepatitis B core antigen gene is necessary but not sufficient for the initiation of transcription. The final conversion of a quiescent, demethylated gene (gamma globin or hepatitis B core antigen) to an active state requires some endogenous or exogenous inducing agent, which may be highly specific for any given gene or gene complex. Clonal selection is not responsible for observed changes in gene expression, since we have clearly shown that cells remethylate DNA that has been demethylated by 5-azacytidine treatment.</p> <p>New micro techniques have been developed which enable the analytical quantitation of 5-methylcytosine in less than one microgram of DNA isolated from any source. Thus, the genomic 5-methylcytosine content of normal human bronchial epithelial and pulmonary mesothelial cells has been measured for the first time. These techniques have also provided for the demonstration that chemical carcinogens can induce decreases in DNA 5-methylcytosine levels in dividing normal human bronchial epithelial cells.</p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on This Project:

Vincent L. Wilson	Sr. Staff Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

To determine the relationship between the changes in 5-methylcytosine patterns in DNA and the carcinogenesis process. It is known that tumor cells contain altered methylation patterns in some genes and DNA sequences as compared to normal tissue. It is not known, however, if these changes in 5-methylcytosine patterns initiate carcinogenesis, occur during carcinogenesis, or are the result of this multistep process. Studies are being directed to determine the ability of chemical carcinogens to inhibit the formation of 5-methylcytosine. These susceptible DNA sequences may be the same areas observed to be undermethylated in tumor cells. The methylation patterns of human tumor DNAs will be probed in the search for demethylated genes and/or DNA sequences that may be specific for the tumor type or tissue of origin.

Methods Employed:

This laboratory has developed and utilized human bronchial tissue and epithelial cell culture as a model for carcinogenesis studies. This system also provides a model for the study of the effects of chemical carcinogenesis on the methylation patterns in the DNA of normal human epithelial cells. DNAs are isolated from carcinogen-treated epithelial cultures; restricted with Hpa II, Msp I, and other enzymes sensitive to cytosine modification; and probed with specific DNA sequences and genes. The genomic levels of 5-methylcytosine will also be monitored in treated cells by a sensitive ³²P post-labeling technique developed in this laboratory. The time course of these effects will also be followed, since previous work has determined that the genomic level of 5-methylcytosine in some mammalian cells is decreased maximally by 48 hours post carcinogenic treatment. Epithelial cell DNA methylation patterns will be compared to those of various carcinoma cell lines and human tumors. High molecular weight DNAs will be isolated from human tumors; subjected to the same enzyme restriction, gel electrophoresis, as above; and probed for alterations in methylation patterns in specific genes and DNA sequences.

Major Findings:

Recent findings have determined not only that methylation patterns in DNA are important to gene expression, but also that changes in these patterns take place during differentiation and in vitro senescence. Thus, the ability of chemical carcinogens to alter 5-methylcytosine patterns in DNA may provide clues to the carcinogenic action of these agents. Previous studies have determined that the alkylation of DNA by alkylating carcinogens inhibits the enzymatic modifications of cytosine residues. Some aromatic hydrocarbon carcinogens also initiated decreases in genomic 5-methylcytosine levels in BALB/3T3 cells.

Previously, the determination of genomic 5-methylcytosine levels required the labeling of DNA in dividing cells with 6-³H-uridine. Limitations in epithelial cell numbers required toxic levels of tritium in order to sufficiently label the DNA for 5-methylcytosine measurements. We have now developed a new method which is both sensitive and does not require active DNA synthesis and cell division. DNA from any source can be enzymatically digested to nucleotides and labeled with ³²P. The labeled nucleotides are then separated by TLC and the ratio of 5-methylcytidine to the total cytidine and 5-methylcytidine determined. This highly sensitive ³²P post-labeling method not only enables the above-described chemical carcinogenesis studies to be performed on human epithelial cells but also allows for monitoring of genomic 5-methylcytosine levels in tumors, tissues, and cell types from human and animal sources. Thus, changes in 5-methylcytosine levels during differentiation and during the normal aging process in vivo can now be followed.

Two separate studies have suggested that only one or two Hpa II methylation sites are important to the expression of selective genes. The human gamma globin gene in mouse erythroleukemia cells containing the human chromosome 11 was found to require the conversion of a few 5-methylcytosines to unmethylated cytosine residues at Hpa II sites in the 5' leading sequences in order to be in an "allowable" state for gene expression. Subsequent treatment of the hypomethylated cells with hexamethylene bisacetamide (HMBA) was required, however, to induce active gamma globin expression in these cells. HMBA is a known inducer of globin synthesis and differentiation of erythroid cells and has been shown to alter the configuration of chromatin. Thus, the methylation pattern may be the first level of regulation of gene expression. The conversion of the quiescent gene to an active state may require demethylation followed by some endogenous or exogenous inducing agents.

This has been further supported by the finding that the expression of the transfected HBV core antigen gene in the carcinoma cell line required both the loss of methylation and subsequent cell divisions in a proper medium. The components in the medium necessary for core antigen expression are not yet known.

Publications:

Wilson, V.L., Smith, R.A., Autrup, H., Krokan, H., Musci, D.E., Le, N.-N.-T., Longoria, J., Ziska, D. and Harris, C.C.: Genomic 5-methylcytosine determination by ³²P-postlabeling analysis. Anal. Biochem. 152: 275-284, 1986.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05326-04 LHC																												
PERIOD COVERED October 1, 1985 to September 30, 1986																														
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) HLA Antigens: Structure, Function and Disease Association																														
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 30%;">PI: Dean L. Mann</td> <td style="width: 30%;">Section Chief</td> <td style="width: 20%;">LHC</td> <td style="width: 20%;">NCI</td> </tr> <tr> <td colspan="4" style="padding-top: 10px;">Others: William Blattner</td> </tr> <tr> <td></td> <td>Chief, Family Studies Section</td> <td>EEB</td> <td>NCI</td> </tr> <tr> <td></td> <td>Shari Bale</td> <td>EEB</td> <td>NCI</td> </tr> <tr> <td></td> <td>Medical Officer</td> <td>EEB</td> <td>NCI</td> </tr> <tr> <td></td> <td>James Geodert</td> <td>EEB</td> <td>NCI</td> </tr> <tr> <td></td> <td>Expert</td> <td></td> <td></td> </tr> </table>			PI: Dean L. Mann	Section Chief	LHC	NCI	Others: William Blattner					Chief, Family Studies Section	EEB	NCI		Shari Bale	EEB	NCI		Medical Officer	EEB	NCI		James Geodert	EEB	NCI		Expert		
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TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 0.5	OTHER: 0.5																												
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SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.) <p>HLA typing was performed on lymphocytes from patients with a common disease or from families where more than one individual had a common disease type. HLA typing was performed in a cohort of individuals with AIDS, either Kaposi's sarcoma or opportunistic infections or individuals at risk for this disease. A total of 250 individuals have been HLA typed. These patients have been followed over a 3-year period. The objectives of these studies are to examine possible genetic susceptibility to the development of AIDS or AIDS-related complex that is related to expression of histocompatibility antigens. Increases in HLA-A3, Aw 23, and Cw 4 were found in patients who developed Kaposi's sarcoma compared to other groups tested. HLA-B8 and DR3 were decreased in frequency and patients with Kaposi's sarcoma were compared to other HTLV-III seropositive individuals. HLA typing was performed in 11 families with hereditary cutaneous malignant melanoma and dysplastic nevi in order to determine if susceptibility was genetically linked to the major histocompatibility complex. No linkage was found. This data is in contrast to previous reports of family studies where it was suggested that the development of melanoma was related to HLA antigens. HLA typing was performed on families where more than one individual had Hodgkin's disease. These studies show a high degree of correlation between the presence of HLA DQ1 antigen homozygosity in individuals who have the disease. These studies suggest the possibility that this locus contributes to the development of this malignancy.</p>																														

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Dean L. Mann	Chief, Biochemical Epidemiology Section	LHC	NCI
William Blattner	Chief, Family Studies Section	EEB	NCI
Shari Bale	Medical Officer	EEB	NCI
James Geodert	Expert	EEB	NCI

Objectives:

To determine function, structure and disease association of major histocompatibility complex genes and/or their products. The studies are directed at the elucidation of genetic associations and potential genetic control of the immune response as it relates to disease process and etiology. Once markers are identified in the disease population, functional and biochemical studies are being performed in order to clearly define the genetic regulation of disease processes as it relates to immunologic response.

Methods Employed:

Standard HLA typing was performed using microcytotoxicity techniques. The technique for HLA-A,B,C has been described by Amos and Poole. The method for typing of the B lymphocytes for HLA-DR determinants was originally described by Mann et al. A total of 19 determinants controlled by the HLA-A locus, 26 alloantigens at the B locus, 6 alloantigens at the C locus, 10 alloantigens at the DR locus and 6 MT antigens were tested for in the population study. HLA typing was performed by an NCI support contract. The association of HLA types with disease was examined for significance by statistical methods.

Major Findings:

This project continues to provide significant information relevant to expression of histocompatibility antigens their genetic control and relationship to disease. HLA typing of the cohort of HTLV seropositive homosexuals, some of whom have developed Kaposi's sarcoma or opportunistic infection in the last three years, revealed some HLA associations with the development of the disease. HLA AW24 is decreased in frequency in the HTLV-III seropositive individuals who do not have Kaposi's sarcoma. HLA-A3, A23, and CW4 antigens are increased in frequency in patients with Kaposi's sarcoma compared to other groups tested. HLA-B8 and DR3 antigens are decreased in frequency in Kaposi's sarcoma compared to other HTLV-III seropositive individuals. HLA CW5 was absent in patients with AIDS. HLA-DRW11 is decreased and HLA-DRW52 increased in frequency in HTLV seropositive individuals compared to seronegative individuals. These preliminary observations provide a possible genetic association for the development of not only AIDS, but of seropositivity to the HTLV-III retrovirus. These studies will continue to assess the frequency of development of individuals with this disease in an attempt to define or predict genetic susceptibility to HTLV-III infection and developing AIDS. HLA typing of the families with malignant melanoma and dysplastic nevus syndrome demonstrated quite clearly that there was no linkage between the combined cutaneous malignant melanoma/dysplastic nevus trait and HLA. In addition, we analyzed data from 19 families that have previously been

reported to have demonstrated some association with HLA antigens. These data were combined and no definitive association of HLA antigens was found. HLA typing of the families with Hodgkin's disease demonstrated homozygosity of the HLA DQ1 locus in individuals with the disease. In comparing these results to incident cases, that is random or sporadic cases, it was found that this same association did not exist in the latter population. Thus, these results suggest strongly the possibility that HLA DQ1 locus antigen plays a role in either the pathogenesis or susceptibility to disease. Expression of HLA antigens controlled by the ABC loci (Class I) and those controlled by HLA DR region antigen (Class II) were determined on normal bronchial epithelial cells and compared to cells freshly explanted from patients with small cell lung carcinoma and the small cell lung carcinoma cells in culture. Relatively normal levels of the HLA class I antigens were found in the bronchial epithelial cells, while both the freshly explanted and the cultured small cell carcinoma cells had low levels of class I antigens. The HLA class II antigens were essentially negative in the normal bronchial epithelial cells; however, in the small cell lung carcinoma tumors, the antigen controlled by this locus was increased to a modest degree. These studies, taken together with other determinations, suggest that histocompatibility antigens may be altered in cells that have undergone malignant transformation.

Publications:

Bale, S. J., Greene, M. H., Murray, C., Goldin, L. R., Johnson, A. H., and Mann, D.: Hereditary malignant melanoma is not linked to the HLA complex on chromosome 6. Int. J. Cancer 36: 439-443, 1985.

Hemler, M. E., Jacobson, J. G., Brenner, M. B., Mann, D. and Strominger, J. L.: VLA-1: A T cell surface antigen which defines a novel late stage of human T cell activation. Eur. J. Immunol. 15: 502-508, 1985.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05328-04 LHC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Immunologic Studies of Human T-Cell Lymphoma Virus		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Dean L. Mann	Section Chief LHC NCI
Others:	Mikulas Popovic	Medical Officer LTCB NCI
	Marvin Reitz	Medical Officer LTCB NCI
	Robert Gallo	Chief LTCB NCI
	William Blattner	Chief, Family Studies Section EEB NCI
	Jeffrey Clark	Senior Staff Fellow EEB NCI
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Human Carcinogenesis		
SECTION Biochemical Epidemiology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 1.0	OTHER:
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>The human T-cell lymphoma virus, HTLV-I, has been found to be associated with patients with adult T-cell leukemia. Studies are underway to understand the mechanism of malignant transformation of cells infected with this virus and the immunologic response of individuals who are infected with this virus that demonstrate malignancies or those who are carriers of the virus but have not developed malignancies. Monoclonal antibodies were developed against protein products produced by a murine cell line which had been transfected with the recombinant plasmic clone containing HTLV-I proviral DNA sequences in regions coding for part of the env px and 3'LTR. Monoclonal antibodies reacted with different patterns when tested by Western blotting on proteins derived from purified HTLV-I and HTLV-II particles. The predominant pattern observed was binding into a p21 and p43 glycopeptide from both HTLV-I and/or HTLV-I, -II, and -III. Experiments were carried out to examine the effect of purified human HTLV-I, -II, and -III retroviruses on normal human lymphocytes of PHA-induced blastogenesis. The inactivated virus induced exaggerated concanavalin A proliferation and suppressed the PHA response. The purified virus causes inhibition of blastogenesis by PHA as did the virus preparations from HTLV-I, -II, and -III. B-cell chronic lymphocytic leukemia (CLL) cells were obtained from patients who were HTLV seropositive, however, whose malignant B-cells did not contain the HTLV-I retrovirus. Using hybridoma technology CLL cells were fused with a B lymphoblastoid cell line and the immunoglobulin captured. In one instance the captured immunoglobulin reacted with the HTLV-I p24 gag proteins and in the other instance the large envelope protein from HTLV-I. Immunoglobulin gene rearrangement present in the B CLL cells was demonstrated in the hybridoma cell line. The results indicate that the CLL cells were antigen committed cells prior to malignant transformation.</p>		

PROJECT DESCRIPTIONNames, Titles, laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Dean L. Mann	Chief, Biochem. Epidemiology Sect.	LHC	NCI
Mika Popovic	Medical Officer	LTCB	NCI
Marvin Reitz	Medical Officer	LTCB	NCI
William Blattner	Chief, Family Studies Section	EEB	NCI
Jeffrey Clark	Senior Staff Fellow	EBB	NCI

Objectives:

These studies were designed to examine the biologic effects of infection with the HTLV-I retrovirus. A number of studies from our lab and other labs have demonstrated that HTLV-I infection altered the immunologic response effecting the dynamics of help and suppression. We have initiated investigations to study the nature of this suppressive effect using the purified retrovirus and to assess alterations in lymphocyte blastogenesis. HTLV-I infection occurs in individuals with other neoplasms. The retrovirus has been found to be absent in neoplastic cells other than in those individuals who have the T-cell lymphomas. Experiments have been carried out to examine the possibility that HTLV-I infection was indirectly involved in the pathogenesis of malignancies other than the specific transforming event of the HTLV-I infection.

Methods Employed:

HLA typing was carried out using alloantisera detecting 16 alleles at the A locus, 26 alloantigens of the B locus, and 6 alloantigens of the HLA-C locus. HLA-DR and MT antigens were detected by alloantisera detecting 10 determinants in the series. Other cell surface markers were examined using the fluorescence-activated cell sorter and monoclonal antibodies directed against cell surface determinants that define subpopulations and/or specific functional subsets of lymphocytes. Indirect immunofluorescence was employed in these studies. Cell lines were established from patients with human T-cell lymphoma virus infection and/or those individuals with the associated adult T-cell leukemia. The cultures were established using PHA and T-cell growth factor and maintained in the presence of T-cell growth factor. The exception that was observed was the spontaneous growth of the B-cell lines, which were demonstrated to be infected with HTLV. Both HTLV-I and HTLV-II were transferred into recipient cells by coculture techniques. The donor cells were irradiated and placed in culture with the recipient cell line. The cells were assayed for the presence of the HTLV gag proteins, p19 and p24, periodically. RNA and DNA were prepared from cells by the standard methodologies. DNA and RNA were probed with the HTLV nick-translated probes.

Major Findings:

Murine monoclonal antibodies were developed against protein products produced by the murine C127 cells which have been transfected with their recombinant plasmid clone containing a portion of the human T-cell leukemia virus Type I (HTLV). This plasmid containing part of the envelope px and 3'LTR provided DNA coding regions. Four different antibodies were developed from .

immunization of the transfected cell line into mice. These antibodies had different reactivity patterns. One of the antibodies reacted against HTLV-I infected cells, but not against noninfected cell lines. This antibody also reacted with purified HTLV-I and -II particles with preferential binding to HTLV-I. This antibody bound to two proteins of approximately 21 and 43 kilodaltons in preparation from purified HTLV-I virus using Western blot analysis. This antibody also reacted against the 43 kilodalton protein and cell lysates from the HTLV-I infected cells. No significant binding was found to HTLV-II proteins in the Western blots. Three other antibodies recognized the same size proteins in the Western blotting in viral preparations from HTLV-I, -II, and -III. The identity of the 21 kilodalton viral proteins is most likely a small envelope protein. We have been unable to identify the origin of the larger protein (43 kilodalton). Inactivated preparations of purified human T-cell virus, HTLV-I, -II, and -III, were found to inhibit normal human lymphocytes. Inhibition was concentration dependent.

Lymphocyte proliferation induced by concanavalin A was enhanced markedly by the addition of HTLV-I and -II inactivated viral preparations. Lymphocyte blastogenesis in response to pokeweed mitogen and mitogenic stimulation were not effected in any significant way by viral protein preparation. We conclude from these experiments that the suppressive product is apparently functioning by binding to lymphocyte membranes. HTLV-I C type retrovirus, as well as the related retroviruses HTLV-II and -III, were used to probe lymphocytes from patients with systemic lupus erythematosus. Nucleic acid hybridization studies failed to reveal any HTLV proviral sequences. In addition sera from these individuals were tested for reactivity to HTLV-I or -III. No antibody activity was found. Human T-cell leukemia virus (HTLV-I) seropositive individuals have been observed in patients with B-cell chronic lymphocytic leukemia cells from the West Indies, an area endemic for HTLV-I infection. The CLL cells obtained from two patients were fused with the human B lymphoblastoid cell line resulting in immunoglobulin secreting hybridoma cell lines. The IgM produced by the hybridoma cell lines from one patient reacted specifically with the p24 gag protein from all three types of HTLV retroviruses with preferential reactivity against the HTLV-I. The hybridoma cell line from patient 2 produced an IgM immunoglobulin reacting with HTLV-I infected cell lines only. The specific immunoglobulin gene rearrangement (IgM, k) in the CLL was demonstrated in the hybridoma cell line. The CLL cells appeared to be a malignant transformation of an antigen-committed B-cell responding to HTLV-I stimulation.

Publications:

Blattner, W. A., Clark, J. W., Gibbs, W. N., Williams, C. K. O., Nomura, A., Mann, D., Saxinger, C., Robert-Guroff, M. and Gallo, R. C.: HTLV: Epidemiology and Relationship to Disease. In M. Miwa et al. (Eds.): Retroviruses in Human Lymphoma/Leukemia. Tokyo/YNU Science Press, 1985, pp. 93-108.

Boumpas, D. T., Hooks, J. J., Popovic, M., and Mann, D. L.: Human T-cell leukemia-lymphoma virus I and/or EB virus infected B-cell lines spontaneously produce acid labile alpha interferon. J. Clin. Immunology 5: 340-344, 1985.

Boumpas, D. T., Popovic, M., Mann, D. L., Balow, J. E. and Tsokos, G. C.: Type C retrovirus of the human T-cell leukemia family are not evident in patients with systemic lupus erythematosus. Arthritis Rheum. (In Press)

Clark, J. W., Hahn, B. H., Mann, D. L., Wong-Staal, F., Popovic, M., Richardson, E., Strong, D. M., Loften, W. S., Blattner, W. A., Gibbs, W. N., Gallo, R. C.: Molecular and immunologic analysis of an HTLV-positive CLL case from Jamaica. Cancer 56: 495-499, 1985.

De Rossi, A., Aldovini, D., Franchini, G., Mann, D. L., Gallo, R. C. and Wong-Staal, F.: Clonal selection of T lymphocytes infected by cell-free HTLV-I virus: Parameters of virus integration and expression. Virology 143: 640-645, 1985.

Eiden, M., Newman, M., Fischer, A. G., Mann, D. L., Hawley, P. M. and Reitz, M. S.: HTLV-I small envelope protein is expressed in mouse cells using a papillomavirus-derived shuttle vector. Mol. Cell. Biol. 11: 3320-3324, 1985.

Jarrett, R. F., Mitsuya, H., Mann, D. L., Cossman, J., Broder, S. and Reitz, M. S.: Configuration and expression of the T cell receptor β chain gene in human T-lymphotrophic virus I-infected cells. J. Exp. Med. 163: 383-399, 1986.

Newman, M. J., Baker, I. T., Reitz, M. S., Eiden, M., Blattner, W. A., Gallo, R. C. and Mann, D. L.: Serologic characterization of human T-cell leukemia (lymphotropic) virus, type (HTLV-I) small envelope protein. Virology 150: 106-116, 1986.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05341-04 LHC	
PERIOD COVERED October 1, 1985 to September 1986			
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Model Systems for Studying Physical Carcinogens at the Cellular Level			
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)			
PI:	John F. Lechner	Section Chief	LHC NCI
Others:	Brenda Gerwin	Research Chemist	LHC NCI
	Curtis C. Harris	Chief	LHC NCI
COOPERATING UNITS (if any) Duke University, Department of Pharmacology, Durham, NC (G. Rosen), Baltimore V.A. Hospital, Baltimore, MD (E. Gabrielson), Institute of Occupational Health, Helsinki, Finland (K. Linnainmaa)			
LAB/BRANCH Laboratory of Human Carcinogenesis			
SECTION In Vitro Carcinogenesis Section			
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892			
TOTAL MAN-YEARS: 2.5		PROFESSIONAL: 1.5	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews			
SUMMARY OF WORK (Use standard unraduced type. Do not exceed the space provided.) <p>Methods have also been developed to culture pleural mesothelial cells obtained from noncancerous donors. Pure cultures are initiated by pelleting the mesothelial cells from pleural effusion fluid and inoculating the resuspended cells into dishes containing LHC basal nutrient medium supplemented with serum (3%), hydrocortisone (0.5 µm), insulin (5 µg/ml) epidermal growth factor (EGF) (5 ng/ml), transferrin (10 µg/ml), and trace elements. Using this protocol, mesothelial cell cultures have been established from more than 200 donors. The cells can be subcultured at clonal density with a colony-forming efficiency of more than 10% and high density cultures can be subcultured four to six times before senescence.</p> <p>A serum-free medium formulated on the above medium but without serum and in addition containing TGF-beta and PDGF will induce serum/factor-starved cells to undergo one round of DNA synthesis. The cells will continue growing, though at a slow rate, if the latter formula is supplemented with "cell-derived" human IL-1. We have studied the cytopathology of fibers on human mesothelial cells and human fibroblasts in culture. Electron microscopy studies have demonstrated phagocytosis of the fibers and phase-microscopy of metaphase spreads reveal frequent attachment of the fibers to the chromosomes. Investigations regarding the mechanism of asbestos carcinogenesis suggest that oxygen radicals are probably not important intermediates.</p> <p>Asbestos-induced chromosomal abnormalities have also been studied. After one exposure to amosite, a significant increase in chromosomal aberrations was found in mesothelial cells, as compared to unexposed controls. The number of aberrant cells was further increased in cultures treated twice with asbestos, the major aberration type being dicentric chromosomes and acentric fragments.</p>			

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

John F. Lechner	Chief, In Vitro Carcinogenesis Section	LHC	NCI
Brenda Gerwin	Research Chemist	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

To study the carcinogenicity and cytopathology of asbestos fibers in human mesothelial and bronchial epithelial in vitro systems. These studies include the following: (1) develop defined media for replicative mesothelial cell cultures, (2) evaluate cytotoxicity of asbestos fibers and synthetic nonmineral fibers in mesothelial and bronchial epithelial cells, (3) evaluate the effects of asbestos fibers on progression of chromosome rearrangements in mesothelial cells, and (4) evaluate the putative role of oxygen radicals in the mode of action of asbestos-caused carcinogens.

Methods Employed:

Mesothelial cells are obtained from pleural effusions of donors without cancer. The fluid is centrifuged, and the pelleted cells are resuspended and inoculated into 100 mm culture dishes at a ratio of one dish per 150 ml of pleural fluid. The cells are dissociated using trypsin when the cultures attain subconfluency. The cultures are further expanded and either cryopreserved or used according to experimental protocols.

Several criteria are used to establish the identity of the cells grown in culture, including immunofluorescent staining with antikeratin antibodies; a variable cell morphology depending on the presence (fusiform) or absence (cobblestone) of EGF and hydrocortisone in the growth medium; histochemical staining for hyaluronic acid-mucin; the presence of long, branched microvilli; and normal human karyotype of Giemsa-band metaphases.

Both clonal growth dose-response and radioactive thymidine incorporation (into DNA) assays are used to assess the nutrient/growth factor requirements of normal mesothelial cells.

Fiber cytotoxicity is assessed using clonal growth dose-response assays. Sixty millimeter dishes are inoculated at clonal density. Twenty-four hours later, the medium is replaced with medium containing increasing concentrations of fibers. After 3 days of exposure, the fiber-treated and control cultures are rinsed twice with medium, then reincubated in fiber-free medium. Ten days post inoculation, the colonies are fixed in 10% formalin and stained with 0.25% crystal violet.

The protocol for the in vitro carcinogenesis experiments begin with exposing second passage cells to two doses of amosite asbestos (2 µg/ml). The cultures are subcultured and carried as long as possible, looking for evidence of phenotypically altered cells. Concurrent control cultures are carried out in

parallel. For chromosomal preparations the mesothelial cell cultures are first partly synchronized by serum starvation in G1 stage and then released from the block. Exponentially growing cells are then treated with colcemid to accumulate the mitotic cells in metaphase and the harvested cells are treated with hypotonic solution, fixed in acetic acid methanol mixture and dropped on slides. Slides are stained with Giemsa for aberration analyses and trypsin-Giemsa method is applied for the karyotypic analyses.

Intracellular oxygen radicals are measured using electron paramagnetic resonance (EPR) with the spin trap 5,5 dimethyl-1-pyrroline-1-oxide (DMPO) and free radical scavengers (N-acetylcysteine, glutathione, D- α -tocopherol and superoxide dismutase) have been added to media to test for modification of asbestos toxicity. DNA damage by free radical mechanisms is measured by the alkaline elution technique that detects single-strand breaks.

Major Findings:

Growth Control Studies: Factor/serum-starved cells will undergo one round of DNA synthesis if the basal nutrients containing insulin is supplemented with EGF, TGF-beta or PDGF. EGF, TGF-beta and PDGF are of equal potency and no further enhancement of mitogenic activity is obtained by using dual combination of these factors, although the combination of all three is about 3 times more potent than is any one alone. However, these factor supplements do not support sustained mesothelial cell replication unless plasma is also present. The potency of plasma alone to stimulate one round of DNA synthesis is about 2 times the potency of the individual growth factors and the effects of dual combinations of plasma and a growth factor are additive. Only the dual combination of EGF + PDGF with plasma further enhances the rate of DNA synthesis by the cells; TGF-beta has no effect except alone with plasma. Slow but sustained growth occurs with plasma only in combination with EGF and only PDGF significantly enhances the growth rate; e.g., doubling the value obtained by plasma and EGF alone. There is great interindividual variation among mesothelial cells cultures established from different donors as measured by responsiveness to factors including serum and plasma. Further ca. 50% of the cultures will undergo slow but sustained growth in plasma-free medium (containing insulin, EGF, TGF-beta and PDGF if IL-1 is also present. However, the remaining cultures exhibit no mitogenic response to IL-1.

Carcinogenesis Studies with Mesothelial Cells: Two subculturings after amosite asbestos exposure, colonies of phenotypically altered cells were present in all of the cultures. These abnormal-appearing cells were not present in the control cultures. The control cultures reach senescence during the fourth to sixth subculture. However, the amosite-exposed cultures have continued to multiply for more than 19 subsequent subculturings (> 50 population doublings). Tumorigenicity of the abnormal-appearing cells was tested by injecting 11th passage post-second amosite exposure cells s.c. into adult athymic nude mice (5 million cells/mouse, 9-20 mice per experiment); no tumors arose within 18 months post-inoculation. Amosite-exposed cells from subsequent experiments have behaved similarly.

Chromosomal Studies of Asbestos-Exposed Mesothelial Cells: After only one exposure to amosite, a significant increase in chromosomal aberrations including chromosome and chromatid breaks was observed in mesothelial cell cultures, compared to untreated control cells. The number of aberrant cells was further increased in cultures treated twice with asbestos, the major aberration types being dicentric

chromosomes and acentric chromosome fragments (minute chromosomes). The number of hypodiploid cells was observed to increase with the passage number of the culture. No significant differences were found, however, between the asbestos-exposed cultures and the corresponding control cultures. Similar results have been obtained from two repeated experiments. The karyotypic analyses of the cells from the latest passages of the two experiments (passages 3 and 5) are underway. In addition, a significant number of the metaphases exhibit asbestos fibers in close opposition to chromosomes.

Effects of Asbestos on DNA Structure: Human mesothelial cells were exposed to doses of 1, 10, or 100 $\mu\text{g/ml}$ of amosite asbestos and measurements of single-strand breaks (SSB) were made with the alkaline elution technique. Measurements of SSB were also made in asbestos-exposed cells coincubated with the DNA polymerase inhibitor combination of 1- β -D-aribinofuranosylcytosine (AraC) and hydroxyurea (HU). The elution of DNA was slightly increased for the experiments with 10 or 100 $\mu\text{g/ml}$ amosite exposure for 24 hours. The calculated amount of damage in each of these three experiments was less than 1 SSB/10 billion daltons, regarded as an insignificant level of induction of SSB. No enhancement of SSB was seen when cells were exposed to AraC/HU for 4 hours, either simultaneously or with the addition of fibers.

Measurements of Superoxide Anion Release in Amosite-Treated Mesothelial Cells: Measurements of superoxide anion were made using electron paramagnetic resonance (EPR), spectroscopy, and the spin-trap agent 5,5-dimethyl-1-pyrroline-1-oxide (DMPO). Mesothelial cells exposed to high levels (10 $\mu\text{g/ml}$) of amosite in the EPR chamber of mesothelial cells exposed to 10 $\mu\text{g/ml}$) amosite in culture for either 5 or 24 hours immediately prior to and during the measurement resulted in EPR spectra identical to the cell-free control. This contrasted with the formulation of a paramagnetic species observed after incubation of suspended cells with 0.1 mM menadione, a quinone capable of redox cycling. The menadione spectrum observed suggests production of superoxide by the cells under these conditions and serves as a positive control for this experiment; exposure of mesothelial cells to this concentration of menadione for 2 hours does not reduce the colony-forming efficiency. Reduced glutathione (1 mM), D- α -tocopherol (300 mM), N-acetylcysteine (300 mM), and superoxide dismutase (50 mg or 100 $\mu\text{g/ml}$) were added to cultures of human mesothelial cells during asbestos exposure, and the clonal growth rate or colony-forming efficiency was compared to similar cultures not exposed to free-radical modifiers. The concentrations of glutathione, D- α -tocopherol, and N-acetylcysteine were previously determined to be the maximal concentrations not inhibitory to mesothelial cell growth. None of these agents altered the toxic effects of asbestos on the mesothelial cell cultures.

Publications:

Gabrielson, E. W., Rosen, G. M., Grafstrom, R. C., Strauss, K. E. and Harris, C. C.: Studies on the role of oxygen radicals in asbestos-induced cytopathology of cultured human lung mesothelial cells. Carcinogenesis. (In Press)

Lechner, J. F., Tokiwa, T., LaVeck, M. A., Benedict, W. F., Banerjee, A., Banks-Schelegel, S. P., Yeager, H. Jr. and Harris, C. C.: Asbestos-associated chromosomal changes in human mesothelial cells. Proc. Natl. Acad. Sci. USA. 82: 3884-3888, 1985.

Lechner, J. F., Tokiwa, T., Yeager, H. Jr. and Harris, C. C.: Asbestos-associated chromosomal changes in human mesothelial cells. In Beck, E. G. and Bignon, J. (Eds.): Third International Workshop in the In Vitro Effects of Mineral Dusts. Berlin, Springer-Verlag, 1985, pp. 197-202.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05403-03 LHC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Molecular Analysis of Gene Regulation and Proliferative Control in Human Cells		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Brenda I. Gerwin	Research Chemist LHC NCI
Others:	Roger Reddel	Guest Researcher LHC NCI
	John Lechner	Res. Microbiologist LHC NIC
	Tohru Masui	Visiting Scientist LHC NCI
	Peter Wirth	Expert LHC NCI
	Snorri Thorgeirsson	Chief LHC NCI
	Anita Roberts	Research Chemist LC NCI
	Michael Sporn	Chief LC NCI
COOPERATING UNITS (if any) Litton Bionetics, Rockville, MD (M. Valerio); Institute of Occupational Health, Helsinki, Finland (K. Linnainmaa)		
LAB/BRANCH Laboratory of Human Carcinogenesis		
SECTION Carcinogen Macromolecular Interaction Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>These experiments have shown that human mesothelial cells as compared to fibroblasts are more sensitive to induction of structural chromosomal aberrations by exposure to asbestos fibers. In addition, it has been shown that malignant mesothelioma cell lines produce TGF-beta and PDGF-2 chain message while normal primary mesothelial cell cultures respond to mitogenic stimuli from the protein products of these messages. These findings suggest the possibility of an autocrine mechanism for the generation of mesothelioma.</p> <p>Two dimensional gel analysis of normal human bronchial epithelial cells after TPA or TGF-beta treatment has indicated several protein alterations which might correlate with squamous differentiation. The magnitude of the alterations is not great, implying that this technique may not display the most critical changes. It is of interest that Northern blot analysis indicates that a 2 hour treatment of human bronchial epithelial cells with TPA but not TGF-beta can induce an increase in IL-1 beta.</p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Brenda I. Gerwin	Research Chemist	LHC	NCI
Roger Reddel	Guest Researcher	LHC	NCI
John Lechner	Res. Microbiologist	LHC	NCI
Tohru Masui	Visiting Associate	LHC	NCI
Peter Wirth	Expert	LEC	NCI
Snorri Thorgeirsson	Chief	LEC	NCI
Anita Roberts	Research Chemist	LC	NCI
Michael Sporn	Chief	LC	NCI

Objectives:

The goal of this project is to understand, at the molecular level, regulatory interactions of human cells with growth factors and metabolites which govern their growth potential and differentiation status. The topics of present interest are: to establish critical differences between normal human mesothelial cells and human mesothelioma cell lines, to study the effects of asbestos on human mesothelial cells as compared to human fibroblasts and to identify the nature of the regulatory pathways in terminal differentiation of human bronchial epithelial cells.

Methods Employed:

Normal mesothelial cells, asbestos-modified mesothelial cells and malignant mesothelioma cells are grown in tissue culture as are normal human bronchial epithelial cells. Whole cell RNA or genomic DNA is prepared from these cells and from fresh or frozen tissue samples. Whole cell lysates labelled with radioactive amino acids are utilized for studies of protein production. Nucleic acids are studied by blotting and probing with appropriate genetic probes. Proteins are studied by immune precipitation and electrophoresis or by two-dimensional gel electrophoresis. Metaphases are prepared from asbestos-treated cells or normal control cells and analyzed both for numerical and structural chromosomal aberrations.

Major Findings:

Acute Effects of Asbestos Exposure: Dose response studies performed on human mesothelial cells and human bronchial fibroblasts showed that treatment with either one or two micrograms per ml of amosite asbestos induced structural aberrations in mesothelial cells but not in fibroblasts. In addition, it was noted that subtoxic concentrations of asbestos induced a significant degree of aneuploidy in both cell types with the population skewed towards hypodiploidy.

Passage Effects: Asbestos-treated and control cultures of normal human mesothelial cells were passaged until senescence and observed at each passage for development of structural and/or numerical chromosomal aberrations. Structural chromosomal aberrations were induced acutely both by exposure to asbestos and Code 100 glass fibers. Since highly damaged cells did not survive, the number

of aberrant cells decreased with passage number. In contrast, control cultures did not develop significant structural aberrations until they became senescent. Numerical aberrations were not significantly induced by these doses of asbestos. An increase in aneuploid cells was observed near senescence in both treated and control cells.

Growth Factor Studies: We have determined that EGF, TGF-beta, and PDGF can independently initiate a single round of DNA synthesis in normal human mesothelial cells. Measurement of TGF-beta and PDGF-2 chain(sis) RNA expression has shown that TGF-beta production is higher in mesotheliomas than in normals and that PDGF-2 chain RNA is expressed only in the tumor cell lines. Measurements of mitogenic activity in conditioned medium have confirmed these results by indicating that the mesothelioma cell lines secrete TGF-beta and PDGF-like activity into their culture medium. In addition, we have recently shown that IL-1 beta is produced by a mesothelioma cell line but not by a normal human mesothelial cell.

Bronchial Epithelial Cells: We have detected the expression of IL-1 beta in normal bronchial epithelial cells and have shown that it is induced by TPA but not by TGF-beta. Both of these agents induce squamous differentiation.

Two dimensional gels have been utilized to detect quantitative changes in proteins produced by human bronchial epithelial cells after treatment with TGF-beta or TPA for various times. These analyses indicated that significant change occurred but that the magnitude of the alterations was not large, generally falling between 1.5-and 2.5-fold changes. After 24 hours, TPA and TGF-beta induced a total of 26 alterations, 6 of which were common. After 72 hours, 42 alterations were observed with only 9 being common to both agents. Equilibrium labelling with inorganic pyrophosphate detected no alterations with TPA or TGF-beta treatment.

Publications:

None

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05408-03 LHC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Drug Metabolism Phenotyping of Nonhuman Primates		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: Vincent L. Wilson Sr. Staff Fellow LHC NCI Others: Curtis C. Harris Chief LHC NCI Susan M. Sieber Deputy Director DCE NCI		
COOPERATING UNITS (if any) Department of Pharmacology, St. Mary's Hospital, London, England (J.R. Idle)		
LAB/BRANCH Laboratory of Human Carcinogenesis		
SECTION Biochemical Epidemiology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 0.5	PROFESSIONAL: 0.2	OTHER: 0.3
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Phenotyping of animal models and man for their xenobiotic metabolizing capabilities has, in recent years, been undertaken in the interests of predicting carcinogenic susceptibilities. The metabolism of exogenous agents is known to be genetically dependent, and selective in vivo xenobiotic metabolic routes appear to be accessible to evaluation by the use of nontoxic doses of certain drugs. The determination of the rate of selective enzymatic modifications of test agents may thus provide a suggestion as to how susceptible an individual may be to the oncogenic potential of carcinogens activated by similar metabolic routes. Several agents including debrisoquine, S-mephenytoin, S-carboxymethyl-L-cysteine, and sulfamethazine have been shown to be metabolized by enzymatic routes governed by separate genetic loci. Thus, these agents will be used to phenotype nonhuman primates on the basis of their abilities to metabolize these compounds. Since the primate colony from which these monkeys will be sampled has been and is presently involved in ongoing chemical carcinogenesis experiments, the results of the metabolic phenotyping can be compared to the susceptibilities of the monkeys to carcinogenesis.		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Vincent L. Wilson	Sr. Staff Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
Susan M. Sieber	Deputy Director	DCE	NCI

Objectives:

To determine the relationship between the genetically dependent rates of selective enzymatic reactions and the chemical carcinogenic susceptibilities in nonhuman primates, man's phylogenetically closest relative. Previous work has demonstrated that chemical carcinogens are activated by selective enzymatic routes in vitro and in rodent animal models. Some correlation has been found between the rates of activation of some carcinogens and the carcinogenic susceptibilities of rats as well. Several studies have clearly demonstrated human polymorphisms in the rates of some of these metabolic routes. However, the correlation of these human metabolic phenotypes with cancer susceptibility has to be based solely on epidemiological data. Thus, more phenotyping data are needed from animal models phylogenetically closer to man than rodents and where carcinogenesis data are available.

Methods Employed:

Subefficacious doses of debrisoquine (DBQ), S-mephenytoin (MPH), S-carboxymethyl-L-cysteine (SCMC), or sulfamethazine will be administered p.o. to monkeys, and 24-hour urine samples collected and analyzed for parent drug and metabolite(s). The methods for quantitation of these compounds in urine have been previously reported in human studies. These four agents were chosen because they each represent a metabolic phenotype governed by separate genetic loci. The enzymatic reactions monitored by these agents are aliphatic ring hydroxylation by DBQ, aromatic ring hydroxylation by MPH, S-oxidation by SCMC, and N-acetylation by sulfamethazine.

The results of phenotyping will be compared with the known carcinogenic susceptibilities of these monkeys.

Major Findings:

Preliminary results of phenotyping three monkeys from each of three species, rhesus, cynomolgus, and African green monkeys, are available. All of the monkeys tested rapidly metabolized DMQ, MPH, and SCMC. The metabolic ratio (concentration of parent drug to metabolite) was observed to range from 0.02 to 0.6 for DBQ, which is well below that reported for man. No differences between species were observed with the minimal sample size of three monkeys per group. MPH metabolism was extensive in these monkeys as well. From 6% to 52% of the administered dose was excreted as 4 hydroxy-MPH in 24 hours, which is greater than that reported for man. There were no significant differences in the rate of MPH hydroxylation between species. The rate of sulfoxidation of SCMC was also high in these monkeys. The metabolic ratio ranged between 0.8 and 3.8, but the rhesus and cynomolgus monkeys metabolized

SCMC significantly ($P < 0.10$) faster than the African green monkeys. The average SCMC sulfoxidation index per species was 1.4 ± 0.3 , 1.2 ± 0.3 , and 2.7 ± 0.9 for rhesus, cynomolgus, and African greens, respectively. These rates are more rapid than that reported for man. Rhesus monkeys were significantly ($P < 0.01$) slower N-acetylators of sulfamethazine than African green monkeys, while the rate in the cynomolgus monkeys fell between the former two species. The average rate of N-acetyl-sulfamethazine formation was $51 \pm 4\%$, $65 \pm 12\%$, and $76 \pm 9\%$ for rhesus, cynomolgus, and African green monkeys, respectively. This rate of sulfamethazine acetylation in rhesus monkeys compares well with previously reported values. These rates also compare well with the human data, classifying the rhesus as poor N-acetylators, the African greens as extensive N-acetylators, and individual cynomolgus monkeys in both categories.

The rapid rate of enzymatic hydroxylation observed in these monkeys for DBQ and MPH would suggest that these monkeys would be susceptible to carcinogenesis upon exposure to aromatic hydrocarbon carcinogens. Aflatoxin B₁ and methyl-azoxymethanol-acetate did produce tumors in these species (Adamson and Sieber in Langenbach, R., Nesnow, S. and Rice, J.M. [Eds.]: Organ and Species Specificity in Chemical Carcinogenesis, New York, Plenum Press, 1983, pp. 129-140). However, several other carcinogens, including benzo[a]pyrene, 3-methylcholanthrene, and cigarette smoke condensate, did not induce tumors. Thus, there may be some correlation between the metabolic phenotype and chemical carcinogenesis susceptibility, but the data presently available are insufficient to draw firm conclusions.

Publications:

Mitchell, S. C., Idle, J. R., Autrup, H., Harris, C. C., Waring, R. H., Ritchie, J. C., Crothers, M. J., Sieber, S. M. and Wilson, V. L.: Sulphoxidation of S-carboxymethyl-L-cysteine in rhesus monkey (Macaca mulatta), cynomolgus monkey (Macaca fascicularis), African green monkey (Cercopithecus aethiops) and the marmoset (Callithrix jacchus). J. Compar. Biochem. Physiol. (In Press)

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05409-03 LHC																									
PERIOD COVERED October 1, 1985 to September 30, 1986																											
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Control of Growth and Differentiation of Human Bronchial Epithelial Cells																											
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 40%;">PI: Tohru Masui</td> <td style="width: 30%;">Visiting Associate</td> <td style="width: 15%;">LHC</td> <td style="width: 15%;">NCI</td> </tr> <tr> <td colspan="4">Others:</td> </tr> <tr> <td>John F. Lechner</td> <td>Section Chief</td> <td>LHC</td> <td>NCI</td> </tr> <tr> <td>Curtis C. Harris</td> <td>Chief</td> <td>LHC</td> <td>NCI</td> </tr> <tr> <td>LaLage M. Wakefield</td> <td>Visiting Fellow</td> <td>LC</td> <td>NCI</td> </tr> <tr> <td>Michael B. Sporn</td> <td>Chief</td> <td>LC</td> <td>NCI</td> </tr> </table>				PI: Tohru Masui	Visiting Associate	LHC	NCI	Others:				John F. Lechner	Section Chief	LHC	NCI	Curtis C. Harris	Chief	LHC	NCI	LaLage M. Wakefield	Visiting Fellow	LC	NCI	Michael B. Sporn	Chief	LC	NCI
PI: Tohru Masui	Visiting Associate	LHC	NCI																								
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LaLage M. Wakefield	Visiting Fellow	LC	NCI																								
Michael B. Sporn	Chief	LC	NCI																								
COOPERATING UNITS (if any) Univ. of MD School of Medicine, Balt., MD (B.F. Trump); Georgetown Univ. School of Medicine, Washington, DC (H. Yeager); VA Hospital, Washington, DC (P. Schafer)																											
LAB/BRANCH Laboratory of Human Carcinogenesis																											
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INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892																											
TOTAL MAN-YEARS: 2.2	PROFESSIONAL: 1.2	OTHER: 1.0																									
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SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Defined methods to grow replicative cultures of normal human bronchial epithelial (NHBE) cells without serum have been developed. These cells can be subcultured several times; will undergo 35 population doublings; and have expected epithelial cell characteristics of keratin, desmosomes and blood group antigens on their cell surface. NHBE cells inoculated at clonal density will multiply with an average generation time of 28 hr; the majority of the cells are small and migratory and have few tonofilaments. Adding human whole blood-derived serum (BDS) depresses the clonal growth rate of NHBE cells in a dose-dependent fashion. In contrast, 10 representative lines of human lung carcinomas either replicate poorly or fail to grow at all when inoculated at clonal density in serum-free medium. Their rates of multiplication increase in direct proportion to the amount of BDS added to the optimized medium. BDS reduces the clonal growth rate of NHBE cells by specifically inducing squamous differentiation. The differentiation-inducing activity was not present in plasma but was found in platelet lysates. Type beta transforming growth factor (TGF-beta) was found to be the primary differentiation-inducing factor in serum for NHBE cells, while TGF-beta was not growth inhibitory for malignant cells. These differential effects of TGF-beta on normal versus malignant cells are not because of lack of TGF-beta-specific receptors on malignant cells. Epinephrine antagonized the effect of TGF-beta without altering characteristics of TGF-beta-specific receptors.																											

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on This Project:

Tohru Masui	Visiting Fellow	LHC	NCI
John F. Lechner	Section Chief	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
LaLage M. Wakefield	Visiting Fellow	LC	NCI
Michael B. Sporn	Chief	LC	NCI

Objectives:

To develop systems to study mechanisms involved during malignant transformation of human epithelial cells. These studies include the following: (1) develop defined media for replicative epithelial cell cultures from bronchial tissues; (2) develop efficient assays to quantify the various squamous differentiation-inducing factors; (3) identify and characterize a factor in BDS that induces squamous differentiation; (4) identify and characterize an autocrine growth factor; (5) identify and characterize an autocrine squamous differentiation-inducing factor; (6) elucidate the pathways of squamous differentiation and determine aberrations that cause human lung carcinoma cells not to respond to these squamous differentiation-inducing factors; (7) elucidate the growth response of normal epithelial cells and determine aberrations that induce differences in growth factor dependencies of human lung carcinomas.

Methods Employed:

Human bronchial tissues are obtained from a medical examiner and "immediate" autopsy donors. Bronchial tissues are dissected from surrounding stroma, cut into 0.5 cm square pieces and used to establish explant cultures. Replicative cultures of normal bronchial epithelial cells are developed from explant culture outgrowths. Upon transfer of the explants to new dishes, the outgrowth cultures remaining in the original dishes are incubated in defined serum-free medium to expand the population and are then subcultured. These normal human bronchial epithelial (NHBE) cells are used in growth and differentiation studies or are cryopreserved for future use. Mitogenicity is quantified by measuring the clonal growth rate, rate of incorporation of tritiated-thymidine into acid precipitable material, and the labeling index by autoradiography. Squamous differentiation is determined by measuring the cell area, extra cellular plasminogen activator activity, Ca ionophore-induced cross-linked envelope formation. Since epinephrine antagonizes the effect of TGF-beta, LHC-8 (LHC-9 medium without epinephrine and retinoic acid) is used mainly in the experiments.

Major Findings:

Human bronchial epithelial cell culture experiments have yielded the following results. A method for routinely initiating replicative epithelial cell cultures of human bronchus was developed. Large pieces of bronchus tissue were initially set up as explant cultures and incubated in a rocking chamber for 7-10 days to facilitate reversal of ischemia. The explants were then cut into smaller pieces, explanted and incubated in a serum-free medium optimized

for growth of NHBE cells. This medium (LHC-9) permits rapid outgrowth of epithelium but retards growth of the fibroblastic cells. The medium is a modification of MCDB 152. The major changes are as follows: the concentrations of arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, valine, choline and serine are doubled; the concentrations of Mg ion and Ca ion are increased 3.5 times; the concentrations of NaCl and HEPES buffer are reduced 20%. LHC-9 is supplemented with insulin, epidermal growth factor (EGF), transferrin, phosphoethanolamine, triiodothyronine, epinephrine, retinoic acid, bovine pituitary extract, and gentamicin.

Several markers that can be used as quantitative assays for squamous differentiation-inducing activity were determined as the following: (1) increase in cross-linked envelope formation; (2) increase in cell area; (3) increase in extracellular plasminogen activator activity; (4) decrease in clonal growth rate; (5) irreversible inhibition of tritiated-thymidine incorporation.

Supplementation of LHC-8 with as little as 1% fetal bovine or human whole blood-derived serum (BDS) resulted in a decrease in clonal growth rate; 8% supplementation completely inhibited growth by inducing terminal squamous differentiation. Human lung carcinoma lines were also incubated in LHC-8 medium with and without 8% BDS. The results showed that serum toxicity per se was not responsible for the observed inhibition of NHBE cell growth; all 10 carcinoma lines divided significantly more rapidly ($p < 0.05$) in BDS-supplemented medium. Thus, the carcinoma cells have both increased requirements for BDS mitogens and a greatly reduced ability to respond to a factor in BDS that induces the normal cells to undergo squamous differentiation. Immunoperoxidase staining for involucrin clearly revealed that NHBE cells exposed to BDS were arranged in a multilayered fashion. The overlying cells were large and strongly involucrin positive, whereas the basal cell sheets were involucrin negative.

Type beta transforming growth factor (TGF-beta) isolated from human platelets was studied as the serum factor responsible for inducing NHBE cells to undergo squamous differentiation. NHBE cells were shown to have high affinity receptors for TGF-beta. TGF-beta induced the following markers of terminal squamous differentiation in NHBE cells: (1) increase in Ca ionophore-induced formation of cross-linked envelopes; (2) increase in extracellular activity of plasminogen activator; (3) irreversible inhibition of DNA synthesis; (4) decrease in clonal growth rate; (5) increase in cell area. The IgG fraction of anti-TGF-beta antiserum prevented both the inhibition of DNA synthesis and the induction of differentiation by either TGF-beta or BDS. Therefore, TGF-beta is the primary differentiation-inducing factor in serum for NHBE cells. On the other hand, TGF-beta did not inhibit growth of human lung carcinoma cell lines and v-Ha-ras oncogene transfected NHBE cells in the serum-free monolayer culture system. TGF-beta-specific receptor assay revealed that the differential effects of TGF-beta on NHBE cells and human lung malignant cells are not because of lack of TGF-beta receptors on malignant cells. Epinephrine-antagonized TGF-beta induced inhibition of DNA synthesis and squamous differentiation of NHBE cells without altering characteristics of TGF-beta-specific receptors on NHBE cells. Although epinephrine increased cyclic AMP levels in NHBE cells, TGF-beta did not alter the cyclic AMP levels in NHBE cells either in the presence or absence of epinephrine. Therefore, the action of epinephrine on TGF-beta effect appears to be via

indirect mechanisms. Furthermore cholera toxin and pertussis toxin, both could induce high levels of cyclic AMP in cells through GTP binding protein systems by different mechanisms, caused the similar antagonistic effect on TGF-beta.

Cell density was found to influence the effect of Ca ion on growth. Whereas optimal growth occurred at clonal densities in medium containing 1 mM Ca ion, rapid squamous terminal differentiation occurred when the medium of dividing high-density cultures was changed from 0.1 to 1 mM Ca ion. These observations suggest that the Ca ion concentration influences the activity of an autocrine squamous differentiation-inducing factor. This autocrine differentiation-inducing factor may be TGF-beta, because TGF-beta is a very potent differentiation inducer (50% inhibitory dose for DNA synthesis is 0.4 pM in LHC-8 medium) and also has been found at high concentrations in conditioned media in differentiation-inducing conditions.

Since BDS appeared to induce more complete differentiation than TGF-beta alone, though TGF-beta plays a key role in BDS, we are trying to identify cofactors in differentiation induction of NHBE cells.

That this putative factor may be IL-1 is reinforced by its known calcium ionophore properties and two recent observations. First, adding recombinant IL-1-beta will induce the cells to undergo terminal squamous differentiation. Second, NHBE cells express IL-1-beta mRNA as assayed by Northern blot analysis. These preliminary observations still need to be confirmed. Synergism of these two factors is now under investigation.

Publications:

Harris, C. C., Yoakum, G. H., Lechner, J. F., Willey, J. C., Masui, T., Gerwin, B., Schlegel, S. and Mark, G.: Growth, differentiation and neoplastic transformation of human bronchial epithelial cells. In Harris, C. C. (Ed.): Biochemical and Molecular Epidemiology of Cancer. New York, A.R. Liss. (In Press)

Krokan, H., Lechner, J., Krokan, R. H. and Harris, C. C.: Normal human bronchial epithelial cells do not show an adaptive response after treatment with N-methyl-N'-nitrosoguanidine. Mutation Res. 146: 205-209, 1985.

LaVeck, M. A. and Lechner, J. F.: Isolation and culture of normal human bronchial epithelial cells from autopsy tissue. In Freshney, R. E. (Ed.): Culture of Animal Cells, 2nd Edition, New York, A. R. Liss. (In Press)

Lechner, J. F. : Replicative cultures of human prostatic epithelial cells. In Coffey, D. (Ed.): Assessment of Current Concepts and Approaches to the Study of Prostate Carcinoma. New York, A. R. Liss. (In Press)

Lechner, J. F. and LaVeck, M. A.: A serum-free method for culturing normal human bronchial epithelial cells at clonal density. J. Tissue Cult. Methods 9: 43-48, 1985.

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Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05410-03 LHC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Hepatitis B Virus Carcinogenesis		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: G. H. Yoakum Senior Staff Fellow LHC NCI		
Others: Dean Mann Section Chief LHC NCI Vincent Wilson Senior Staff Fellow LHC NCI Curtis C. Harris Chief LHC NCI		
COOPERATING UNITS (if any) Tsang-Tang Sun, Cancer Institute, Chinese Academy of Medical Science; I-C. Hsu, University of Maryland School of Medicine; B. E. Korba, Division of Molecular Virology and Immunology, Georgetown University, Washington, DC		
LAB/BRANCH Laboratory of Human Carcinogenesis		
SECTION Carcinogen Macromolecular Interaction Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 8.0	PROFESSIONAL: 3.5	OTHER: 4.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) The protoplast fusion method for transfection of human cells was used to construct a human epithelial cell line carrying the hepatitis B virus (HBV) core gene (HBc) stably integrated into the genome (GTC2). The plasmid pKYC200 used to construct GTC2 contains the HBc structural gene and its regulatory elements without other HBV genes. Therefore, GTC2 cells provide an in vitro model system to determine the effects of HBc gene expression without interference from other HBV genes. The cytopathological effects observed during the maximal expression of the HBc gene product provide an insight into the role HBc regulation plays in the pathology of HBV infection. The regulation of HBc expression by the methylation of an Hpa II site 280 bp upstream of the AUG encoding the start site of the HBc structural gene provides the first proof for site-specific regulation of a human virus gene by methylation of 5'-methylcytosine. The detection of replicative forms of HBV in lymphocytes from chronic active hepatitis (CAH) and acquired immunodeficiency syndrome disease (AIDS) and the stimulation of HBc gene expression when GTC2 or PLC/PRF/5 cells are treated with 100 units of alpha interferon (α -IFN) suggest that HBV may have a cytolytic effect during the infection of lymphocytes that is important to the immunological abnormalities frequently associated with HBV infection.		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

George H. Yoakum	Senior Staff Fellow	LHC	NCI
Vincent Wilson	Senior Staff Fellow	LHC	NCI
Dean Mann	Section Chief	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

The role of hepatitis B virus in human hepatocellular carcinoma is being studied in vitro by investigating the direct mechanism of HBV pathology and determining the regulation and biological effects of the hepatitis B virus core antigen gene (HBc) in human cells. This research program focuses on (1) the mechanisms of HBV pathology and its role in carcinogenesis and (2) the mechanism of biological responses involved in these carcinogenic processes with potential application to cancer epidemiology and disease prevention.

Methods Employed:

We have developed a method to genetically transfect a variety of human cell types, including normal fibroblastic and epithelial cells, carcinoma cells, transformed fibroblasts, and lymphoid cell lines. Introduction of HBV and HBV genes into human cells is essential to the development of effective research programs to study the role of HBV in human carcinogenesis at the genetic and molecular levels because there is no system available at this time to infect cells with HBV in tissue culture. The protoplast fusion transfection procedure permits transient expression of transferred genes in 70-90% of the recipient cell cultures for 6-12 days after the procedure. This permits the construction of human cell lines carrying HBV for in vitro characterization of virus pathology and carcinogenic potential. The protoplast fusion method of transfection for transfer of plasmids stably transfers genes into human cells at frequencies greater than 10^{-3} units.

Standard nucleic acid hybridization analysis methods will be utilized to characterize the genetic organization of HBV transfected into human recombinant cell lines constructed for these studies. This includes slot-blot hybridization of DNA or RNA products to detect genomic DNA inserts and characterize transfected gene transcription products. Southern hybridization analysis of the genetic organization of HBV transfectants permits interpretation of gene expression experiments and testing of the methylation state of the transfected virus genome.

Major Findings:

The importance of 5'-methylcytosine to HBc gene regulation was first indicated by the cytotoxicity that follows induction of HBcAg expression in GTC2 cells or in an HBV-containing hepatocellular carcinoma cell line [PLC/PRF/5] after treatment with 5'-azacytidine. The methylation state of Hpa II-280 and Hpa II⁴⁷⁹ was determined by digestion of DNA from nuclei of GTC2 cells with BAM HI or Ava I to release the transfected HBc gene. The DNA was subsequently digested with Hpa II before separation of the fragments by gel electrophoresis for

Southern hybridization. Evaluation of site-specific methylation in the HBc gene of GTC2 cells after treatment with 5'-azacytidine is possible because the ratio of the HBV-hybridizing DNA fragments varies directly with the methylation state at the relevant Hpa II sites. Internalization of the Hpa II sites being tested for methylation by digestion of cellular DNA with Bam HI or Ava I provides an internal control for measurement of methylation at these sites without interference from fragmented HBc gene inserts or from partially digested restriction fragments. This is because the fragments selected for analysis can only be produced by Hpa II digestion of complete HBc gene segments that do not contain inserted sequences. The stoichiometric ratio of specific GTC2 DNA fragments was determined by densitometric analysis of autoradiographic films from Southern hybridization of GTC2 DNA fragments probed with ^{32}P -labeled HBV DNA. By comparing the quantity of specific sizes of HBV-hybridizing DNA fragments with the sizes predicted from the transfected HBc gene sequence in various states of methylation at Hpa II sites, the methylation state at Hpa II-280 and Hpa II+479 in GTC2 DNA was determined. Treatment of GTC2 cells with 5'-azacytidine enhances production of HBcAg. The cytotoxic effect of HBc induction in GTC2 cells follows an initial increase in HBcAg production at four to six divisions after cells are treated with 5'-azacytidine (primary culture, P_0). This increase reaches a maximum at approximately eight to ten divisions after treatment (P_1).

The surface antigen and the core antigen (HBcAg) have different roles during HBV infection. Serologic data suggest that the constitutively regulated gene for HBsAg (HBs) is needed for expansion of the focus of viral infection because antibodies to HBsAg are required for protection against the virus by immunization and are present during recovery from infection. In contrast, antibodies to the HBV core gene (HBc) product do not appear consistently during recovery and are frequently associated with virus replication and consequent infectivity of patients sera. To understand the mechanism of HBV pathology during acute and chronic disease processes, it is essential to separate the various viral genetic elements and to study their biological effects and molecular biology in a model cell system in vitro. This project demonstrates the value of such an approach by revealing the importance of the core antigen in the cytotoxic response of cells to infection with HBV, a role previously unassigned to any specific HBV gene. The methylation state of chromosomal DNA has been implicated as a general controlling factor in carcinogenesis. The system described here provides a unique opportunity to study, at the molecular level, the role of DNA cytosine methylation in controlling the expression of a specific gene of established biological importance in human cells. HBV infection of lymphocytes from patients with AIDS and chronic active hepatitis (CAH) is detectable by nucleic acid hybridization analysis. The infection of immune system cells by HBV may be important in the pathogenesis and immunological abnormalities associated with these conditions.

Publications:

Korba, B. E., Wells, F., Tennant, B. C., Yoakum, G. H., Purcell, R. H., and Gerin, J. L.: Hepadnavirus infection of peripheral blood lymphocytes in vivo: the woodchuck and chimpanzee models of viral hepatitis. J. Virol. 58: 1-8, 1986.

Yoakum, G. H., Harris, C. C., Korba, B. E., Boumpas, D. C., Mann, D. L., and Sun, T.-T.: The molecular biology of human hepatitis B virus infection. J. Cell. Biochem. (In Press)

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05424-02 LHC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) The Role of Respiratory Viruses in Lung Carcinogenesis		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	C. C. Harris	Chief LHC NCI
Others:	R. R. Reddel	Guest Researcher LHC NCI
	Y. Ke	Visiting Fellow LHC NCI
	B. I. Gerwin	Research Chemist LHC NCI
	J. Rhim	Microbiologist LCMB NCI
	J. B. Park	Visiting Fellow LCMB NCI
COOPERATING UNITS (if any) Dept. of Immunology and Infectious Disease, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, MD (C. Christian, K. Shah).		
LAB/BRANCH Laboratory of Human Carcinogenesis		
SECTION Carcinogen Macromolecular Interaction Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 2.0	PROFESSIONAL: 1.75	OTHER: 0.25
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) This project is to assess the role of viruses in the multistage process of human lung carcinogenesis, using normal bronchial epithelial and mesothelial cells. Immortalized, nontumorigenic clonal cell lines have been derived from these cell types via viral infection (SV40 virus, BK virus, or adenovirus-12--SV40 hybrid virus) or transfection of viral genes (SV40 T-antigen gene-, or SV40 genome-containing plasmids). Some of the bronchial epithelial cell lines retain the ability to undergo terminal squamous differentiation in response to serum. These cells are being used for studies of the effects of other DNA tumor virus genes, other oncogenes, and carcinogens.		

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

C. C. Harris	Chief	LHC	NCI
R. R. Reddel	Guest Researcher	LHC	NCI
Y. Ke	Visiting Fellow	LHC	NCI
B. I. Gerwin	Research Chemist	LHC	NCI
J. Rhim	Microbiologist	LCMB	NCI
J. B. Park	Visiting Fellow	LCMB	NCI

Objectives:

To study the effects of viruses on normal human mesothelial and bronchial epithelial cells in vitro; to utilize virally transformed cells for chemical and physical carcinogen studies investigating multistage carcinogenesis; to analyze cooperative effects between genes of DNA viruses and other oncogenes.

Methods Employed:

This project employs several techniques developed in this laboratory including culture of normal human bronchial epithelial cells from explanted autopsy tissue and mesothelial cells from pleural effusions, and transfection of these cells with DNA. Selected viral genes have been subcloned into the pSV₂ neo-vector. Virally infected and transfected cells have been monitored primarily by phase-contrast light microscopy and also by indirect immunofluorescence techniques. Viral shedding from transformed cells was assayed by co-cultivation with Vero cells. Cellular transformation has been detected by colony or focus formation, morphological changes, and senescence escape; and transformed cell lines characterized by karyotype analysis, electron microscopy, and injection into nude athymic mice.

Major Findings:

No transformation has been seen, to date, following infection with a range of human influenza and parainfluenza viruses, respiratory syncytial virus, coxsackie B3 virus, JC virus, human papillomavirus 1, and adenoviruses 5 and 12.

Clonal cell lines have been derived from both human mesothelial and bronchial epithelial cells by infection with SV40 virus or adenovirus-12-SV40 hybrid virus. An uncloned mesothelial cell line has been produced by BK virus infection. Both cell types were also transformed by transfection with plasmids containing the SV40 T-antigen gene or the whole SV40 genome. Many of the transformed bronchial epithelial cell lines retain the ability of the cell of origin to undergo terminal squamous differentiation in the presence of serum. All of the lines have been shown to have human isozymes and karyotype analysis has shown that all of the lines are aneuploid. Indirect immunofluorescence has detected expression of T-antigen and keratin in all lines, and in the case of the mesothelial lines, vimentin also. Injection of transformed cells into irradiated athymic nude mice has not yet resulted in the appearance of any tumors after periods ranging from 1-9 months.

Conditions have been established for transfection of transformed cells with plasmid DNA, and selection with G418 following transfection with pSV₂neo-derived plasmids. Experiments have begun to assess the effects in these cells of other DNA tumor virus genes, other oncogenes, and growth factor genes. In addition, chemical and physical carcinogens are being used either before or after T-antigen gene transfection in a study of multistep carcinogenesis.

Publications:

None

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05426-02 LHC	
PERIOD COVERED October 1, 1985 to September 30, 1986			
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Characterization, Mode of Action, and Evolution of the Oncogene <u>raf</u>			
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: George E. Mark, III Expert LHC NCI			
Others: Andrea Pfeifer Visiting Fellow LHC NCI Paul Amstad Visiting Fellow LHC NCI Dean L. Mann Section Chief LHC NCI Curtis C. Harris Chief LHC NCI Maria G. Tsokos Visiting Scientist LP NCI Nicolae C. Popescu Microbiologist LB NCI			
COOPERATING UNITS (if any) Clinical Neuroscience Branch, NIMH, NIH (C. B. Pert); Dept. of Anatomy, Case Western Reserve University (N. Perrimon); V.A. Medical Center, Syracuse, NY (S. Graziano).			
LAB/BRANCH Laboratory of Human Carcinogenesis			
SECTION Carcinogen Macromolecular Interaction Section			
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892			
TOTAL MAN-YEARS: 0.7		PROFESSIONAL: 0.5	
		OTHER: 0.2	
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews			
SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.) We have investigated the expression of the <u>c-raf-1</u> proto-oncogene in neoplastic and hyperplastic pathologies, as well as in normal tissues. All small cell lung carcinomas examined express elevated levels of <u>raf</u> transcript. Characterization of surface markers revealed the presence of early monomyelocyte antigens on cultured SCLC cell lines as well as on cells obtained from fresh biopsies. The expression of these and other surface workers is altered following treatment with gamma interferon; surprisingly, some cells began expressing T-cell markers following treatment. Employing an anti-peptide antiserum directed toward v- <u>raf</u> /c- <u>raf</u> shared determinants, the distribution of <u>raf</u> in normal rat brain was examined. Specific binding was observed which in some areas represented fiber tracts, while in other areas neuron bodies were stained. The distribution seen corresponds to specific cytoarchitectural sites: ventral thalamus, hippocampus, caudate putamen, septum, entorhinal cortex, inferior colliculus, and cerebellar nuclei. Regions representing the limbic system are especially enriched for the <u>raf</u> polypeptide. The pattern is that of a widely disseminated neuropeptide receptor. Two neural crest-derived malignancies (Ewing's sarcomas and neuroepitheliomas) have been found which express <u>raf</u> while neuroblastomas express significant amounts of <u>raf</u> RNA and protein only following cAMP induced differentiation. We have constructed two vectors containing either the complete <u>raf</u> cDNA sequence or the anticomplementary 3' domain obtained from the preceding sequence for the purpose of 1) identifying <u>raf</u> 's endogenous ligand and 2) ascertaining its role in proliferation by observing the consequences of specifically inhibiting its translation in SCLC and neuroepithelioma cells. Finally, the new proto-oncogene (pks) sequence we previously characterized has been mapped by somatic cell hybrids and in situ hybridization to Xp11.2-11.4. A related sequence was also found on chromosome 7, near the centromere.			

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on This Project:

George E. Mark, III	Expert	LHC	NCI
Andrea Pfeifer	Visiting Fellow	LHC	NCI
Paul Amstad	Visiting Fellow	LHC	NCI
Dean L. Mann	Chief, Biochem. Epidemiol. Section	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
Maria G. Tsokos	Visiting Scientist	LP	NCI

Objectives:

Of the four major classes of oncogenes the largest class contains the members of the src family. These genes, many of which have been shown to possess either a tyrosine or serine/threonine kinase activity, are found to be associated with the cell membrane and are believed to be components of the cell's receptor system. It is the stimulation of one or more of these receptors which initiates a cascade of events leading to cellular proliferation. The objectives of this project are (1) to identify and characterize new proto-oncogenes so that additional potential targets of transforming lesions might be enumerated along with loci involved in normal cellular growth and differentiation, and (2) to determine what human disorders may involve the inappropriate expression of the proto-oncogenes related to raf in the hope that the pathology may illuminate the physiologic role of these genes.

Methods Employed:

RNAs were isolated from tissue culture cells, or human peripheral neuroepithelioma cells by the guanidinium isothiocyanate-CsCl method of Chargwin. Analysis of RNAs was either quantitative dot blotting or qualitative Northern analysis followed by hybridization with nick-translated 32-P labelled probes. Nucleic acid sequencing protocols, described for dideoxy sequencing from primer initiated plasmids, were employed. Surface marker studies were performed on the FACS using established fluorescent labelled monoclonal antibody procedures. Published methods for immunofluorescent staining of fixed cells and radioimmune localization of peptides from frozen sections were employed.

Major Findings:

The c-raf-1 proto-oncogene, a unique member of the src family as a consequence of its intrinsic serine (not tyrosine) kinase activity, has been localized to the short arm of human chromosome 3, proximal to deletions observed in most small cell lung carcinomas. Cells derived from this particularly aggressive type of lung cancer have been shown to contain amplified expression of one of the myc proto-oncogenes (either c-myc, N-myc, or L-myc) as well as significant levels of c-raf-1 expression. Does this explain their malignant phenotype.

All of the SCLC cell lines tested were positive for raf expression, most showing levels of expression ≥ 20 -fold than that found in normal bronchial epithelial cells. This amplified state of transcription was not dependent upon the existence

of an amplified myc proto-oncogene, since no significant difference was found in raf expression in these cells relative to cells in which myc was not amplified. RNAs isolated from fresh lung parenchyma or normal liver obtained by autopsy, or nonsmall cell lung carcinomas obtained by biopsy, revealed little, if any, raf expression. The only normal tissue to consistently show raf transcripts was of lymphoid origin.

To determine the role of raf in the proliferation of SCLC cells, as well as neuroepithelioma cells (see below), vectors capable of introducing all or part of the c-raf-1 sequence into human cells have been constructed. For the introduction of a sequence into essentially all cells under scrutiny, a second generation retroviral vector (pLJ) has been obtained from R. Mulligan (Whitehead Institute, Cambridge, MA). Into the unique Ban HI cloning site of this vector we have inserted both the complete c-raf-1 cDNA sequence (in both orientations) or the 3' terminal 1.1-Kb of this sequence in the anti-sense orientation. This vector has also been used to clone an activated form of the human c-myc gene, again in both orientations. We have found that the introduction of these recombinants into psi-2 cells generates clones producing a high titer of ecotropic oncogene-carrying virus (> 100,000 infectious units per ml). The host range of the vector has then been changed by using this virus to infect psi-AM cells. The amphotropic virus released following this infection is of sufficient titer (> 10,000 infectious units per ml) to infect a 35mm dish of normal human bronchial epithelial (NHBE) cells or human malignant cells. NHBE cells are being infected with raf-, myc-, or raf- and myc-containing viruses to determine the biologic consequences. Human SCLC cells and peripheral neuroepithelioma cells are being infected with the anti-sense versions of these viruses in the hope that translational arrest of each gene product in these malignancies will indicate the roles played by these sequences. Should the level of anti-sense RNA made by infected cells be insufficient to terminate the translation of these genes, an additional series of anti-sense recombinants have been constructed in a vector (pADMLP) utilizing, for transcription, the adenovirus major late promoter which is at least 1000 times more effective in human cells than a murine retrovirus promoter. These recombinants will be introduced by microinjection.

The observation of significant raf expression in lymphoid cells led us to investigate the surface markers on the established SCLC cell lines. Employing fluorescence-conjugated monoclonal antibodies and the FACS, the surface characteristics of various cell lines were determined and compared to fresh tumor tissue obtained from two patients (S. Graziano). The macrophage/monocyte antigen detected by OKM1 has been previously shown to be on the surface of SCLC cells (Ruff and Pert, *Science* 225:1034-1036, 1985) and this observation was interpreted to point toward a myeloid or hemopoietic stem cell as the target of transformation. The unique presence of two early monomyelocytic markers, MY4 and MY9, on the SCLC cells obtained from cultured lines as well as fresh biopsies was observed. Several surface characteristics could be altered following the addition of gamma interferon (5000 units/ml), but not the MY4 and MY9 expression. One of the lines investigated actually expressed a T-cell marker (OKT3) as a consequence of gamma interferon addition. It is tempting to conclude that small cell lung carcinomas are, in actuality, lymphomas whose stem cell was an immature hematopoietic cell.

In collaboration with Dr. Pert's laboratory (NIMH) affinity-purified raf-specific antibodies were employed to examine rat brain for the existence and distribution of this polypeptide. Specific binding was observed with antisera specific for different regions of the raf molecule. This reactivity was present on fiber tracts in some regions, while in other areas the neuron bodies were positive. The distribution seen corresponds to specific cytoarchitectural sites: ventral thalamus, hippocampus, caudate putamen, septum, entorhinal cortex, inferior colliculus, pontine nuclei and cerebellar cortex and nuclei. Regions representing the limbic system are especially enriched for the raf polypeptide. The pattern is that of a widely disseminated neuropeptide receptor. We have found raf to be potentially associated with embryonic and neural development in *Drosophila* (see below), suggesting that its physiologic role may be as a neuropeptide receptor rather than as a lymphoid receptor shared by neuronal cells.

We sought to determine if raf could be found associated with any malignancy involving neuronal cells. In collaboration with Dr. Tsokos (DCBD) we find higher raf expressed in Ewing sarcomas (stem cell believed to be undifferentiated neural crest cells) and peripheral neuroepitheliomas than in neuroblastomas. However, neuroblastomas are induced to express raf following cAMP-initiated axonal differentiation. Thus, raf expression in these cells appears to be differentiation stage specific.

In an attempt to identify the endogenous ligand for the c-raf-1 polypeptide we are placing the aforementioned retroviral c-raf-1 construct into mouse 3T3 cells (since these latter cells respond to the raf kinase signal by proliferating). Neuropeptides contained within brain homogenates will be screened for the ability to induce proliferation in the c-raf-1-infected cells.

Most proto-oncogenes have been highly conserved through evolution since the appearance of metazoan organisms; hence, the belief that their role in normal cellular development and differentiation has transgressed time unaltered. A *Drosophila melanogaster* genomic library, made from size-selected partial EcoRI digested DNA ligated into the gamma phage Charon 4A, had been screened with a 32-P labelled v-raf probe under relaxed stringency conditions to identify the Draf1 locus. The predicted amino acid sequence of Draf1 indicates it is a raf homolog and as such probably provides identical functions in fruit flies as the related genes do in man and mouse. The localization of this raf homolog to 2F5,6 on the *Drosophila* genome has led to its exact location, via DNA walking, to 2F6 by N. Perrimon. In collaboration with us they are manipulating this sequence by P element vectoring and mutagenesis to determine its function in *Drosophila* embryogenesis.

The initial screening of a human fetal liver cDNA library for raf homologs was performed under conditions of low hybridization stringency. Two nonidentical clones were isolated from this gamma gt 10 library. Restriction enzyme mapping showed one of these clones represented a cDNA copy of a portion of the c-raf-1 gene, while the other was unique. DNA sequencing of this unique gene revealed it was a new raf-related human locus.

To determine the number of pks genes in the human genome, Southern blot analyses were performed on DNAs from mouse x human somatic cell hybrids. Hybridization to restricted human DNA under conditions of high stringency revealed strong hybridization to the three Bgl II fragments which co-segregated, indicating that

they represented the cloned pks gene. The largest Bgl II fragment hybridized poorly under high stringency to the middle and 3' untranslated region probes. We conclude that there are two closely related pks genes in man. Employing extensive analysis of many mouse x human somatic cell hybrids, we have localized the pks gene to the short arm of the X chromosome (Xpter-Xp11) and the other gene to chromosome 7 (7pter-7q22). Exact localization of these two genes was performed by in situ hybridization (N. Popescu) and we examined 122 hybridized cells exhibiting 250 grains. Of these, 71 and 39 were localized to chromosomes X and 7, respectively, jointly representing 44% of all the grains scored; no other specific labeling sites were observed. On the X chromosome, grains were tightly clustered on the short arm with an accumulation of 65 grains in the region Xp11.2-11.4. Specific labeling on chromosome 7 was observed over the region of the centromere (grains were located at the centromere and both proximal and distal to it). From the total grain distribution, we conclude that there are two loci of the pks gene, one at Xp11.2-11.4 representing pks and the other at 7p12-7q11.21 representing a pks-related sequence.

Publications:

Bonner, T. I., Kerby, S. B., Suttrave, P., Gunnell, M. A., Mark, G. and Rapp, U. R.: The structure and biological activity of the human homologues of the raf/mil oncogene. Mol. Cell. Biol. 5: 1400-1407, 1985.

Boumpas, D. T., Mark, G. E. and Tsokos, G. C.: Oncogenes and autoimmunity. Anticancer Res. (In Press)

Boumpas, D. T., Tsokos, G. C., Mann, D. L., Eleftheriades, E. G., Harris, C. C. and Mark, G. E.: Increased proto-oncogene expression in peripheral blood lymphocytes from patients with systemic lupus erythematosus and other autoimmune disease. Arthritis Rheum. (In Press)

Harris, C. C., Yoakum, G. H., Lechner, J. F., Willey, J. C., Gerwin, B., Banks-Schlegel, S., Masui, T. and Mark, G. E.: Growth, differentiation and neoplastic transformation of human bronchial epithelial cells. In Harris, C. C. (Ed.): Biochemical and Molecular Epidemiology of Human Cancer. New York, A. L. Liss. (In Press)

Mark, G. E., Seeley, T. W., Shows, T. B. and Mountz, J. D.: PKS. A new raf related sequence in man. Proc. Natl. Acad. Sci. U.S.A. (In Press)

Mountz, J. D., Mushinski, J. F., Mark, G. E. and Steinberg, A. D.: Oncogene expression in autoimmune mice. J. Mol. Cell. Immunol. 2: 121-131, 1985.

Schultz, A. M., Copeland, T. D., Mark, G. E., Rapp, U. R. and Oroszlan, S.: Detection of the myristylated gag-raf transforming protein with raf-specific antipeptide sera. Virology 146: 78-89, 1985.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05427-02 LHC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Human Chorionic Gonadotropin as a Marker and Growth Factor in Human Lung Tumors		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Douglas Brash	Sr. Staff Fellow LHC NCI
Others:	Curtis C. Harris	Chief LHC NCI
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Human Carcinogenesis		
SECTION Carcinogen Macromolecular Interaction Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 0.5	PROFESSIONAL: 0.5	OTHER: 0.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>HCG secretion by human tumor lines was found to occur in the following pattern: Undifferentiated carcinomas, squamous cell carcinomas, and adenocarcinomas--beta HCG subunit; mucoepidermoid carcinomas--alpha HCG subunit; small cell carcinomas, large cell carcinomas, and mesotheliomas--no HCG. Secretion of beta HCG was highly correlated with activation of the <u>Ki-ras</u> or <u>Ha-ras</u> oncogene at codon 12. Activated <u>myc</u> and <u>raf</u> oncogenes in lung tumors and activated <u>Ki-ras</u> and <u>Ha-ras</u> in nonlung tumors were not correlated with HCG secretion. A method was developed for transfecting calcium-sensitive normal human bronchial cells (NHBE) with plasmid and genomic DNAs, using strontium ion to replace calcium. Transfection of the v-Ki-ras gene or an activated cellular-Ki minigene into NHBE did not result in HCG secretion. Infection with Kirsten sarcoma virus to increase the number of affected cells is in progress. Addition of alpha HCG, beta HCG, or alpha+beta HCG did not increase the growth rate of three human tumor cell lines secreting solely alpha or beta HCG subunits.</p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

D.E. Brash	Sr. Staff Fellow	LHC	NCI
C.C. Harris	Chief	LHC	NCI

Objectives:

To determine whether ectopic HCG production is a consistent marker in human lung tumors. To determine whether α and β subunits of HCG are growth factors for bronchial epithelial cells and lung tumor cells. To determine whether HCG expression is related to oncogene expression, particularly Ki-ras.

Methods Employed:

HCG secretion is measured by radioimmunoassay for α or β subunits. The Ki-61 cell line was derived by outgrowth from minced tumors. Transfection of NHBE cells is by strontium phosphate precipitation (manuscript in preparation). Infection with Kirsten sarcoma virus uses baboon endogenous virus as helper. Cell growth stimulation was measured as increased uptake of tritiated thymidine, with ^{14}C prelabeling as normalization standard.

Major Findings:

1. Twelve of 14 human undifferentiated, squamous cell, and adenocarcinoma cell lines secreted β HCG. Two of these are known to carry activated Ha-ras and five are known to carry activated Ki-ras. Two of two human mucoepidermoid carcinomas secreted α HCG; no oncogene has been reported in these lines. One each of a large cell, small cell classic, and small cell variant carcinoma secreted no HCG, nor did a mesothelioma cell line. Two of two choriocarcinomas secreted large quantities of α and β HCG. In contrast, two bladder carcinoma lines (Ha-ras), two colon carcinoma lines (Ki-ras and myc amplification), one embryonal rhabdomyosarcoma (N-ras), and one promyelocytic leukemia (N-ras; myc amplification) did not secrete HCG. A lung tumor line was derived from a tumor containing Ki-ras activated at codon 61 and its HCG secretion is under examination.

2. To circumvent the calcium-sensitivity of NHBE cells, a DNA transfection procedure was developed in which strontium cation is substituted for calcium. The efficiency of transfection in CV-1 monkey kidney cells was equal to that of calcium phosphate precipitates, and the optimum pH for precipitate formation is broader than that of calcium. Transfection is also obtained with genomic DNA.

3. The Ki-ras oncogene was transfected into NHBE cells as either the v-Ki oncogene under control of an Ha or a Ki LTR or as an activated c-Ki minigene under control of the cellular promoter. No increase in HCG secretion was seen. Since the fraction of cells expressing the transfected gene may have been low, we are currently infecting NHBE with Kirsten sarcoma virus plus baboon endogenous virus as helper to achieve nearly 100% introduction of the gene.

4. To determine whether growth of tumor cells secreting only α or β HCG subunit was stimulated by the missing subunit, HCG subunits were added to sparse cultures of A1146 (α), CaLu-1 (β) and A427 (β). No growth stimulation was seen. A repeat at clonal densities is planned. A similar experiment on NHBE is in progress.

Publications:

None

Patents:

None

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05429-02 LHC															
PERIOD COVERED October 1, 1985 to September 30, 1986																	
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Use of Retroviral Shuttle Vector for Infection of Oncogenes Into Human Cells																	
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 35%;">Curtis C. Harris</td> <td style="width: 20%;">Chief</td> <td style="width: 15%;">LHC</td> <td style="width: 15%;">NCI</td> </tr> <tr> <td>Others:</td> <td>Paul Amstad</td> <td>Visiting Fellow</td> <td>LHC</td> <td>NCI</td> </tr> <tr> <td></td> <td>George E. Mark, III</td> <td>Expert</td> <td>LHC</td> <td>NCI</td> </tr> </table>			PI:	Curtis C. Harris	Chief	LHC	NCI	Others:	Paul Amstad	Visiting Fellow	LHC	NCI		George E. Mark, III	Expert	LHC	NCI
PI:	Curtis C. Harris	Chief	LHC	NCI													
Others:	Paul Amstad	Visiting Fellow	LHC	NCI													
	George E. Mark, III	Expert	LHC	NCI													
COOPERATING UNITS (if any)																	
LAB/BRANCH Laboratory of Human Carcinogenesis																	
SECTION Carcinogen Macromolecular Interaction Section																	
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892																	
TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 1.0	OTHER:															
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews																	
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The introduction of foreign genes into a retroviral shuttle vector offers the opportunity of infecting a variety of human cell types at high frequency. The coding region of the v-Ha-ras oncogene was cloned in both orientations into the murine retroviral shuttle vector pZip-neo SV(X). Plasmid DNA of bacterial clones containing the Ha-ras coding region was isolated and transfected into NIH 3T3 cells by the calcium phosphate precipitation method. One out of six clones transfected was biologically active in induction of transformed foci.</p> <p>The plasmid DNAs (pZip-neo-ras and pZip-neo-sar orientation) were transfected into Psi-2 cells by the calcium phosphate precipitation method. G418 resistant clones of transfected Psi-2 cells were isolated and their supernatant was titered for virus production. Virus titers of about 10 to the fifth power virus/ml were achieved. Subsequent infection of Psi-am cells with recombinant virus produced from Psi-2 cells yielded virus stocks of 5 x 10 to the third power - 10 to the fourth power virus/ml.</p>																	

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Paul Amstad	Visiting Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
George E. Mark, III	Expert	LHC	NCI

Objectives:

The introduction of foreign genes into a retroviral shuttle vector offers the possibility of infecting a variety of mammalian cells, including human, at a very high frequency. We focus on studying transformation of normal human bronchial epithelial (NHBE) cells and their regulation of differentiation by using the retroviral shuttle vector as a means of effectively transferring genes into human cells.

Methods Employed:

The 1.8 kb Bgl II fragment of clone H1 containing the structural gene of v-Ha-ras was isolated, recessed with Bal 31 to between 0.9 and 1.2 kb in length and then cloned into the unique BamHI site of the Zip-neo SV(X) shuttle vector. Bacterial clones of HB101 containing the recombinant plasmid were identified by colony hybridization using the 1.8 kb Bgl II ras fragment as a probe. Fourteen v-Ha-ras/Zip recombinants were isolated. Eight of those contained the insert in frame orientation relative to the transcription start site within the viral LTR. Six of the eight clones with the desired orientation were tested for biological activity by transfection into NIH 3T3 cells. One clone was found to be positive in the NIH 3T3 transfection assay. v-Ha-ras/Zip and v-Ha-sar/Zip were transfected into Psi2 cells, a mouse cell line containing the integrated form of a packaging deficient mouse MLV providing retroviral functions in trans. G418 resistant clones were isolated and their supernatant was tested for virus production by an infection assay on NIH 3T3 cells. Virus stocks of 10^4 - 10^5 virus/ml supernatant were obtained.

Subsequent infection of recombinant virus produced from Psi2 clones into Yam cells yielded virus stocks of 5×10^3 - 10^4 virus/ml supernatant. The Psi-am viral packaging system provides the recombinant retrovirus with an amphotrophic envelope which enables the virus to infect human cells.

Major Findings:

The structural gene of v-Ha-ras was cloned into the retroviral shuttle vector Zip-neo SV(X). Out of six clones assayed on NIH 3T3, one was shown to be positive in inducing transformed foci. Recombinant virus containing the sense or antisense form of v-Ha-ras were produced at a virus titer of about 10^5 virus/ml supernatant.

Subsequent infection of Psi-am cells with the Psi₂ produced recombinant virus yielded titers of 5×10^3 - 10^4 virus/ml supernatant.

Publications:

None

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05431-02 LHC																				
PERIOD COVERED October 1, 1985 to September 30, 1986																						
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Transfection of Oncogenes into Human Bronchial Epithelial Cells (NHBE)																						
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 40%;">Brenda Gerwin</td> <td style="width: 25%;">Research Chemist</td> <td style="width: 10%;">LHC</td> <td style="width: 10%;">NCI</td> </tr> <tr> <td>Others:</td> <td>Curtis C. Harris</td> <td>Chief</td> <td>LHC</td> <td>NCI</td> </tr> <tr> <td></td> <td>George Yoakum</td> <td>Sr. Staff Fellow</td> <td>LHC</td> <td>NCI</td> </tr> <tr> <td></td> <td>Paul Amstad</td> <td>Visiting Fellow</td> <td>LHC</td> <td>NCI</td> </tr> </table>			PI:	Brenda Gerwin	Research Chemist	LHC	NCI	Others:	Curtis C. Harris	Chief	LHC	NCI		George Yoakum	Sr. Staff Fellow	LHC	NCI		Paul Amstad	Visiting Fellow	LHC	NCI
PI:	Brenda Gerwin	Research Chemist	LHC	NCI																		
Others:	Curtis C. Harris	Chief	LHC	NCI																		
	George Yoakum	Sr. Staff Fellow	LHC	NCI																		
	Paul Amstad	Visiting Fellow	LHC	NCI																		
COOPERATING UNITS (if any)																						
LAB/BRANCH Laboratory of Human Carcinogenesis																						
SECTION Carcinogen Macromolecular Interaction Section																						
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892																						
TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 1.0	OTHER:																				
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) Human subjects</td> <td><input checked="" type="checkbox"/> (b) Human tissues</td> <td><input type="checkbox"/> (c) Neither</td> </tr> <tr> <td><input type="checkbox"/> (a1) Minors</td> <td></td> <td></td> </tr> <tr> <td><input type="checkbox"/> (a2) Interviews</td> <td></td> <td></td> </tr> </table>			<input type="checkbox"/> (a) Human subjects	<input checked="" type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither	<input type="checkbox"/> (a1) Minors			<input type="checkbox"/> (a2) Interviews													
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<input type="checkbox"/> (a2) Interviews																						
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) NHBE cells were transfected with a variety of different oncogenes: <u>raf</u> , <u>v-Ha-ras</u> , a combination of <u>raf</u> and <u>v-myc</u> on the same plasmid and the translocated <u>c-myc</u> frame of the CA46 Burkitt's Lymphoma (BL) cell line. The transfected cells were then selected for resistance to inducers of differentiation by treating them with blood-derived serum (BDS) or TPA. The CA46 translocated <u>c-myc</u> gene was the most effective oncogene in inducing resistance to differentiation in normal human bronchial epithelial (NHBE) cells. The translocated <u>c-myc</u> gene has been further analyzed by testing deletion mutants of the original clone for resistance to differentiation and induction of transformation in both biological assay systems.																						

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Brenda Gerwin	Research Chemist	LHC	NCI
Paul Amstad	Visiting Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
George H. Yoakum	Sr. Staff Fellow	LHC	NCI

Objectives:

To study the role of oncogenes, especially c-myc, in transformation and in the regulation of differentiation in NHBE cells.

Methods Employed:

NHBE cells have been transfected with plasmid constructs containing oncogenes by the protoplast fusion method. Three days after the fusion, the transfected cells were subcultured to 500,000 cells per dish and one day later selected with 2% BDS or 10 nM TPA for about 3 weeks. After one to two weeks, the appearance of clones resistant to inducers of differentiation were observed. Three weeks after the initial treatment, the dishes were fixed and stained with crystal violet, and the colonies were then counted. The selection was done in LHC-8 medium, a defined growth medium which is devoid of epinephrine and retinoic acid which antagonize BDS and TPA in inducing differentiation.

Deletion mutants of the original CA46 c-myc clone were made by removing either the IgH part of the switch region or the second and third exon of c-myc by standard recombinant DNA technique.

Major Findings:

Among the oncogenes tested on NHBE for induction to resistance of differentiation, the translocated c-myc from CA46 cells showed the strongest response. Clones of NHBE cells resistant to inducers of differentiation like BDS and TPA showed an increased growth potential but did not become immortal; neither was their morphology altered.

Publications:

None

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05432-02 LHC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) The Biological Activity of Fecapentaene-12 in Human Tissues and Cells		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Curtis C. Harris	Chief LHC NCI
Others:	Simon M. Plummer	Visiting Fellow LHC NCI
	Tsyochi Kakefuda	Medical Officer LMC NCI
	Hiroshi Imai	Visiting Fellow LMC NCI
	Vincent Wilson	Senior Staff Fellow LHC NCI
COOPERATING UNITS (if any) Microbiological Associates, Inc., Bethesda, MD (R. Curren, L.L. Yang, D. Putman); Dept. Toxicology, Karolinska Institute, Sweden (R. Grafstrom); Dept. Pathology, University of Maryland, Baltimore, MD (A.K.M. Shamsuddin)		
LAB/BRANCH Laboratory of Human Carcinogenesis		
SECTION Biochemical Epidemiology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	2.0	3.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Fecapentaene-12 (fec-12), a possible initiating agent in colon cancer, is cyto- toxic and mutagenic in human fibroblasts. DNA repair-deficient fibroblasts are more sensitive than normal fibroblasts to both these effects, which are dose dependent. Further studies with human fibroblasts have shown that fec-12 is a potent inducer of DNA single strand breaks (SSB) and sister chromatid exchanges (SCE). Accumulation of SSB as a result of inhibition of the polymerase component of the excision repair mechanism suggests that SSB may be mediated in part by DNA repair mechanisms. Autoradiographic techniques have shown that fec-12 is capable of inducing unscheduled DNA synthesis (UDS) in normal human fibroblasts. These results indicate that fec-12 is genotoxic, mutagenic and causes direct DNA damage in human cells. Further support for the hypothesis that fec-12 is an initiating agent in colon cancer comes from the finding that this compound induces transformation in murine Balb 3T3 cells. Plasmid assays investigating the mechanism of fec-12-DNA damage have shown evidence of interstrand DNA cross-links and direct SSB. This is of interest because it is known that DNA cross-linking agents are potent inducers of SCE. Fec-12 induces plasmid mutations in excision repair-deficient AB 1886 (uvra-) but not AB 2463 (recA-) or wild type strains of E. coli. These results suggests that direct DNA damage and DNA excision repair mechanisms are involved in fec-12-induced mutations. The restriction enzyme digest profile in 10% of plasmids isolated from fec-12- induced mutants showed marked differences from control plasmids, indicating that fec-12 can induce DNA rearrangements.		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Curtis Harris	Chief	LHC	NCI
Simon Plummer	Visiting Fellow	LHC	NCI
Tsyochi Kakefuda	Medical Officer	LMC	NCI
Hiroshi Imai	Visiting Fellow	LMC	NCI
Vincent Wilson	Sr Staff Fellow	LHC	NCI

Objectives:

To assess the possible importance of fec-12 in the etiology of human colon cancer by studying (1) genotoxicity and mutagenicity in human cells and tissues; (2) transforming ability in a mammalian cell assay; (3) molecular mechanism(s) of fec-12-induced DNA damage by in vitro assays; (4) fec-12-DNA damage (e.g., DNA adducts) in DNA isolated from human colon epithelium in populations with different risks from colon cancer.

Methods Employed:

Monolayer cultures of human fibroblasts have been used to study the cytotoxicity and mutagenicity of fec-12 in human cells. Fec-12-induced DNA single strand breaks (SSB), unscheduled DNA synthesis (UDS) and sister chromatid exchanges (SCE) have also been measured in this cell culture system by alkaline elution, autoradiography and bromodeoxyuridine incorporation respectively. The ability of fec-12 to induce morphological transformation was studied in monolayer cultures of murine Balb 3T3 cells.

A plasmid DNA cross-linking assay involving agarose gel electrophoresis and electron microscopy has been developed to study the molecular mechanisms involved in fec-12-DNA damage. A plasmid mediated mutation assay has been used to study the molecular changes occurring as a result of fec-12-induced mutations. Plasmid PKG 1820, which contains an ampicillin resistance gene (amp^r) and a galactokinase gene ($gal K$) governed by a promoter (P_{gal}) and a termination sequence (t^o) which prevents expression of the $gal K$ gene, was used in these experiments. Mutants were analyzed by DNA sequencing (Sanger) of the t^o region and restriction enzyme (Tag 1) digest analysis of the entire plasmid.

Major Findings:

Fec-12 is cytotoxic and mutagenic in human fibroblasts. DNA repair-deficient fibroblasts are more sensitive than normal fibroblasts to both these effects. Fec-12 also causes DNA single strand breaks (SSB), unscheduled DNA synthesis (UDS) and sister chromatid exchanges (SCE) in normal human fibroblasts. Fec-12 induces a significant increase in the transformation frequency of murine Balb 3T3 cells in culture. These results indicate that fec-12 is a potent genotoxic agent in normal human cells and support the hypothesis that fec-12 may be an initiating agent in colon cancer.

In vitro studies into the mechanism of fec-12-DNA damage have established that this compound causes interstrand DNA cross-linking and direct DNA SSB. Fec-12 induces

plasmid (PKG 1820) mutations in excision repair-deficient AB 1886 (uvrA⁻) but not AB 2463 (recA⁻) or wild type strains of E. coli. Analysis of these mutants showed no changes in the DNA sequence of the termination region (t^o) of the plasmid but marked changes in the restriction enzyme (Tag 1) digest profile of 10% of plasmids isolated from fec-12-induced mutants of AB 1886. This result indicates that fec-12 induces DNA rearrangements in this plasmid.

Publications:

Plummer, S. M., Grafstrom, R. C., Yang, L. L., Curren, R. D., Linnainmaa, K. and Harris, C. C.: Fecapentaene-12 causes DNA damage and mutations in human cells. Carcinogenesis. (In Press)

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05433-02 LHC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Synchronous Fluorescence Scanning Detection of Aflatoxin Adducts		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: Vincent L. Wilson Senior Staff Fellow LHC NCI		
Others: Dean Mann Chief, Biochem. Epidemiol. Section LHC NCI Curtis C. Harris Chief LHC NCI		
COOPERATING UNITS (if any) Boston Univ., Boston, MA (J.D. Groopman); Univ. of MD School of Medicine, Baltimore, MD, (A.K.M. Shamsuddin); Cancer Inst., CAMS, Beijing, PRC (Sun Tsung-tang); MIT, Boston, MA (S. Tannenbaum)		
LAB/BRANCH Laboratory of Human Carcinogenesis		
SECTION Biochemical Epidemiology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 1.6	PROFESSIONAL: 0.8	OTHER: 0.8
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Synchronous fluorescence scanning and computer linkages for data analysis have been used to characterize the fluorescent properties, limits of detection, contour maps and 3-dimensional images of a series of aflatoxins, their metabolites, and DNA adducts. The power of these fluorescent techniques has been shown by the demonstration that the number of components within a sample can be delineated using fourth derivative computer analysis of 3-dimensional synchronous fluorescence scanning data. Simple methylation of the 9 position of the guanine residue in AFB1-N7-guanine not only did not increase fluorescence, as has been proposed, but also caused a shift in the optimum delta lambda. Thus, these techniques may provide valuable structural determinations of fluorescent carcinogens and DNA adducts.		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project

Vincent L. Wilson	Senior Staff Fellow	LHC	NCI
Dean Mann	Chief, Biochem. Epidemiol. Section	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

To develop fluorescent techniques for detection of minor levels of aflatoxin adducts in DNA and blood proteins. To determine if liver aflatoxin DNA adduct and/or blood protein adduct levels correlate with the incidence of hepatic cancer in susceptible populations. To establish the usefulness of the analysis of aflatoxin DNA adduct levels to the study of the relationship of carcinogen activation and binding to human cancer.

Methods Employed:

DNA treated with AFB₁ in the presence of a microsomal enzyme activating system, liver DNA from AFB₁ treated rats, and human liver DNA from unknown environmental exposure to AFB₁, will be analyzed by synchronous fluorescence scanning on 650-40 Perkin-Elmer fluorescence spectrophotometer linked to the Perkin-Elmer 3600 data station. Further data manipulation will be on the NIH Dec-10 computer system. DNA of known AFB₁ exposure will also be assessed for the levels and pattern of AFB₁-DNA adducts by HPLC.

Major Findings:

Detection and characterization of the fluorescent properties of several aflatoxins, their metabolites, the major DNA adduct AFB₁-N7-guanine, and DNA modified with AFB₁, has been performed. The sophistication of the available fluorescence instrumentation has enabled the sensitive detection and fingerprint analysis of these compounds by synchronous fluorescence scanning (SFS) spectrophotometry. The limit of concentration detectable by SFS for each of these aflatoxins was determined. Although AFB₁-N7-guanine, the major DNA alkylation product of aflatoxin B₁, was readily detectable by SFS, the conversion of this adduct to the ring-opened AFB₁-FAPY product dramatically reduced the detectability of DNA covalently bound aflatoxin. The optimum delta lambda for each compound was also determined, thus providing the spectral characteristics that distinguish each of these hydrocarbons from one another.

The ability to detect and differentiate fluorescent products in biological fluids and DNA may be feasible due to the adaptation of these techniques to 3-dimensional analysis and the production of contour maps. Data collection for 3-dimensional analysis requires only taking a series of SFS scans over a range of delta lambdas. Contour maps and 3-dimensional images are extremely specific to a given compound, much like a fingerprint. Computer-assisted analysis of 3-dimensional data provided fourth derivative determinations which demonstrated the presence of two components in a standard mixture of AFB₁ and AFM₁. SFS computer assisted analysis of biological samples taken where environmental exposure may have occurred, may, therefore, provide determinations

of how many fluorescent components are present and possibly what some of them are.

The quantitiveness of AFB₁ adducts in DNA by SFS analysis has been demonstrated using in vivo treated rats for a model study. The liver DNA from AFB₁-treated rats was used to determine the level of AFB₁ bound to DNA. These values compared well with those determined by radiolabeled AFB₁ used in the dosing of the rats. However, the SFS analysis of numerous human liver DNA samples provided no significant levels of AFB₁ bound to DNA. Thus, the limit of detection for AFB₁-DNA adducts may not be low enough to analyze for environmental AFB₁ exposure.

Methylation of the N9 position of the guanine residue in AFB₁-N7-guanine had been proposed to enhance fluorescence. SFS analysis of AFB₁-N-Me-guanine demonstrated that not only did the optimum delta lambda increase, but the fluorescence of this adduct decreased as compared to the parent structure. Other adduct modifications, such as acetylation and dansylation, will have to be tested to determine if the SFS detection of AFB adducts can be enhanced.

Publications:

Harris, C. C., LaVeck, G., Groopman, J., Wilson, V. L. and Mann, D.: Measurement of aflatoxin B₁, its metabolites and DNA adducts by synchronous fluorescence spectrophotometry. Cancer Res. (In Press)

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER ZO1CP05434-02 LHC																																			
PERIOD COVERED October 1, 1985 to September 30, 1986																																					
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Immunobiology of AIDS and AIDS-Related Diseases																																					
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 45%;">Dean L. Mann</td> <td style="width: 30%;">Section Chief</td> <td style="width: 10%;">LHC</td> <td style="width: 10%;">NCI</td> </tr> <tr> <td>Others:</td> <td>William Blatter</td> <td>Chief, Family Studies Section</td> <td>EEB</td> <td>NCI</td> </tr> <tr> <td></td> <td>J. J. Goedert</td> <td>Expert</td> <td>EEB</td> <td>NCI</td> </tr> <tr> <td></td> <td>R. J. Bigger</td> <td>Medical Officer</td> <td>EEB</td> <td>NCI</td> </tr> <tr> <td></td> <td>Stanley H. Weiss</td> <td>Medical Staff Fellow</td> <td>EEB</td> <td>NCI</td> </tr> <tr> <td></td> <td>R. C. Gallo</td> <td>Chief</td> <td>LTCB</td> <td>NCI</td> </tr> <tr> <td></td> <td>Mika Popovic</td> <td>Medical Officer</td> <td>LTCB</td> <td>NCI</td> </tr> </table>			PI:	Dean L. Mann	Section Chief	LHC	NCI	Others:	William Blatter	Chief, Family Studies Section	EEB	NCI		J. J. Goedert	Expert	EEB	NCI		R. J. Bigger	Medical Officer	EEB	NCI		Stanley H. Weiss	Medical Staff Fellow	EEB	NCI		R. C. Gallo	Chief	LTCB	NCI		Mika Popovic	Medical Officer	LTCB	NCI
PI:	Dean L. Mann	Section Chief	LHC	NCI																																	
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	Stanley H. Weiss	Medical Staff Fellow	EEB	NCI																																	
	R. C. Gallo	Chief	LTCB	NCI																																	
	Mika Popovic	Medical Officer	LTCB	NCI																																	
COOPERATING UNITS (if any)																																					
LAB/BRANCH Laboratory of Human Carcinogenesis																																					
SECTION Biochemical Epidemiology Section																																					
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892																																					
TOTAL MAN-YEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 1.0																																			
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews																																					
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> Acquired immunodeficiency syndrome (AIDS) is characterized by the profound loss of ability to respond to environmental antigens as well as the development of Kaposi's sarcoma. We have studied the percentage of T-cell subsets in patients with the disease and patients at risk for the disease. In a prospective study, we evaluated the total numbers of T4 positive lymphocytes in 86 HTLV-III antibody-positive AIDS-free homosexual men, 19 of whom developed AIDS between June 1982 and 1985. In evaluation of these T-cell subsets, it was found that the highest degree of correlation with development of the disease was with low numbers of T4 positive cells at the time that the studies were initiated. In skin biopsies from patients with Kaposi's sarcoma, AIDS, or individuals who were HTLV-III sero-positive, we have identified the HTLV-III retrovirus in Langerhan cells. Positive biopsies were found in 7 of 40 individuals with Kaposi's sarcoma and/or AIDS-related complex. No positive biopsies were seen in patients with opportunistic infections. In studies of the early events of binding of the HTLV-III retrovirus to T-cells, we have determined that the epitope defined by a monoclonal antibody detecting the T4A antigen on the T4 molecule is the specific receptor for HTLV-III binding. Also involved in the binding is the HLA-DR molecule, while other products of the HLA-D region appear not to be involved in HTLV-III binding. Peripheral blood monocytes, as well as cell lines with monocyte function and characteristics, have been infected with HTLV-III. These monocytes bear the HLA-DR antigen as well as a molecule detected by the OKT4A antibody. Other antibodies detecting epitopes on the T4 molecule were absent from these cells. </p>																																					

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Dean L. Mann	Chief, Biochem. Epidemiology Sect.	LHC	NCI
William Blattner	Chief, Family Studies Section	EEB	NCI
Robert Gallo	Chief	LTCB	NCI
Robert Bigger	Medical Officer	EEB	NCI
Stanley Weiss	Medical Staff Fellow	EEB	NCI
Mika Popovic	Medical Officer	LTCB	NCI

Objectives:

The objective of this investigation is to study the immunobiology of AIDS and AIDS-related diseases. These studies include investigation of the action of the HTLV-retrovirus on cell depletion, i.e., cell death, the specific receptor site for the attachment of the HTLV-III retrovirus and the mechanism of destruction of these helper cells. The studies are directed also at the understanding of the nature of the determinant on the retroviral envelope which attaches to the OKT4 receptor and in addition, an investigation of the immune response of individuals who are infected with this retrovirus who do not develop AIDS or AIDS-related diseases.

Methods Employed:

Monoclonal antibodies detecting the epitopes on the T4 molecule as well as the T8 molecules were used to study lymphocytes from patients with AIDS and male homosexuals who are at risk for the development of AIDS and who are either HTLV-III seropositive or seronegative. Peripheral blood lymphocytes were obtained from these populations of individuals and cryopreserved until used. The cells were thawed, reacted with monoclonal antibodies and fluorescence conjugated rabbit anti-mouse immunoglobulin. After appropriate periods of incubation, the cells were analyzed with the fluorescence activated cell sorter (FACS). All cells showing forward light scatter pattern were analyzed. The percentages of total populations reacting with monoclonal antibodies were determined and analyzed in relationship to epidemiologic parameters. Sera obtained from these same patients were studied for antibody reactivity to the HTLV-III retrovirus. The ratio of binding of the sera to the retrovirus was considered positive when the titers were five times greater than the control population. Specific questionnaires inquiring about the lifestyle and sexual practice of the individual were formulated by the Environmental Epidemiology Branch at the NCI. The results of the data were collected from these questionnaires and correlated with the numbers of T4 and T8 positive lymphocytes in their peripheral blood. Four (4) mm skin punch biopsies were obtained from the buttocks of individuals who were HTLV-III seropositive and without apparent disease, individuals with AIDS-related complex, and individuals with either opportunistic infection or Kaposi's sarcoma, the latter being considered as having diagnostic AIDS. The biopsies were sectioned and stained by immunohistochemical techniques for a T4 and T6 cell surface antigens to determine the presence of Langerhan's cells as well as the presence of HTLV-III infection. The latter was determined using monoclonal antibodies, detecting the P17 HTLV-III gag protein. Additional

biopsies were fixed in the appropriate fashion and scanned by electron microscopy for the presence of virus particles and/or virus budding from cells in the biopsies. Normal peripheral blood lymphocytes were obtained and monocytes isolated by the adherence technique. These monocytes were cultured for 2 weeks and then infected with the HTLV-III retrovirus. The cell surface antigen expression was determined on these cells with and without infection. Several cell lines which have been established and have monocyte/macrophage characteristics were also infected with HTLV-III and the cell surface antigen was assessed with and without infection. The binding of the HTLV-III retrovirus to T cells was studied in the following manner: The uninfected H9 T-cell line was incubated with HTLV-III retrovirus from several different sources (e.g., different strains) for periods of 15, 30, 60, and 120 minutes. The cells were washed and fixed and the cell surface antigens determined using the fluorescence-activated cell sorter. Antibodies used in these studies were directed against different epitopes on the T4 molecule as well as antibodies detecting HLA-DR, DQ, and DP.

Major Findings:

The studies on T-cell subsets were carried out in cooperation with the Environmental Epidemiology Branch in the NCI. Lymphocytes were obtained from selected populations as described above. Questionnaires were designed to attempt to correlate certain environmental cofactors as well as sexual practices that might predispose to the development of disease. One hundred eighty homosexual men representing patients from two physicians' offices in the Washington D.C. area were enrolled in the study. Lymphocytes from these individuals were studied for the presence of T4 positive and T8 positive lymphocytes using the fluorescence activated cell sorters described above. The data obtained from the laboratory analyses was correlated with the patient history and homosexual contacts with individuals of high-risk areas: New York City, San Francisco, or Los Angeles. A positive correlation was found with decreased numbers of T4 positive cells for most individuals who had homosexual contact with individuals from high-risk areas. The population could be stratified into those of high risk, intermediate risk, and those individuals with low risk--the risk factors being contact with other HTLV-III positive individuals. There was a positive correlation of the total number of T4 positive cells and contact with high-risk individuals. We prospectively studied 86 HTLV-III seropositive individuals. Ten individuals developed opportunistic infection and were diagnosed as having AIDS. The risk of AIDS is clearly predicted by the total number of T4 positive lymphocytes at the time of enrollment when at least 43% of the men with the lowest T4 positive quartile (less than 300 cells per ml) developed AIDS compared to 27% in the second quartile; and 11 and 5 percent in the third and fourth quartile, respectively, developed AIDS. Subjects from Manhattan had a higher risk of AIDS, particularly Kaposi's sarcoma, than did subjects in the Washington D.C. area, again identifying the high-risk population for this disease. Other factors which appeared to correlate with the decreased numbers of T4 positive cells were receptive anal intercourse, enemas, methaqualone use and high levels of hepatitis B surface antibody, the latter having what appears to be an association with the development of Kaposi's sarcoma.

In the skin biopsies from patients with AIDS or at risk for AIDS and normal individuals, Langerhan's cells were easily identified using immunohistochemical techniques. These cells are characterized by the presence of the T6

marker as well as antibodies identifying with the T4 marker. Studies have demonstrated quite clearly that the Langerhan's cells function as macrophage-dendritic cells in the skin presenting antigen to T-cells and serving as macrophage-like cells in the skin. HTLV-III infection was identified in 7 of the 40 individuals who were HTLV-III seropositive. Five positive biopsies were found in individuals who had Kaposi's sarcoma and two who had AIDS-related complex. Other HTLV-III seropositive individuals, including individuals with AIDS with opportunistic infections, were negative for the retrovirus in these 4 mm skin punch biopsies. In the one individual in which the HTLV retrovirus was searched for, electron microscopy demonstrated the presence of budding virus as well as viral particles characteristic of the HTLV-III retrovirus. These studies identify a new reservoir for HTLV-III and are of particular interest in that positive biopsies were found in AIDS patients with Kaposi's sarcoma, a malignant disease that is often present in the skin.

Studies of binding of the HTLV-III retrovirus to human T-cells were carried out to determine the specific receptor site for the binding of HTLV-III. In our studies we demonstrated quite clearly that within one-half hour there is binding of the retrovirus to the T4 molecule as demonstrated by the absence of binding antibodies detecting the T4A epitope on this molecule when the virus was present. In parallel with this decrease in the ability to detect T4A antigen, we also found that HLA-DR was also decreased on these cells within 15 to 30 minutes of viral attachment. We compared the presence of HLA-DR with the products of two other loci in the HLA-D region, HLA-DP and DQ. The results indicate clearly that the HLA-DR is involved as a receptor for the HTLV-III retrovirus infection. Peripheral blood monocytes were studied for the expression of cell surface antigens before and after HTLV-III infection. It was found that peripheral blood monocytes express the T4A antigenic determinants, but not determinant, detected by the Leu 3A monoclonal antibody and the T4 monoclonal antibodies. These two latter antibodies detect epitopes that are different from the T4A epitope on the T4 molecule. Peripheral blood mononuclear cells can be divided into those expressing HLA-DR and DQ and/or HLA-DR only. On examination of the cell surface characteristics of monocytes infected with HTLV-III we found that there was a loss of the T4A epitope, an observation that was similar to that observed with T-cells and the expression of HLA-DR only. The results indicate that the T4 molecule is down-regulated in the monocytes which is similar to the observations made in T cells and that the retrovirus most probably selects the population of monocytes which are expressing the HLA-DR molecules only.

Publications:

Goedert, J. J., Bigger, R. J., Melbye, M., Mann, D. L., Wilson, S., Gail, M. H., Grossman, R. J., DiGioia, R. A., Sanchez, W. C., Blattner, W. A.: Modification of AIDS risk by T4 count and cofactors in HTLV-III infected homosexual men. J. Am. Med. Assoc. (In Press)

Goedert, J. J., Bigger, R. J., Winn, D. M., Mann, D. L., Byar, D. P., Strong, D. M., DiGioia, R. A., Grossman, R. J., Sanchez, W. C., Kase, R. G., Greene, M. H., Hoover R. N. and Blattner, W. A.: Decreased helper T-lymphocytes in homosexual men. I. Sexual contact with high incidence areas for the acquired immune deficiency syndrome. Amer. J. Epidemiol. 121: 629-636, 1985.

Goedert, J. J., Bigger, R. J., Winn, D. M., Mann, D. L., Byar, D. P., Strong, D. M., Digioia, R. A., Grossman, R. J., Sanchez, W. C., Kase, R. G., Greene, M. H., Hoover, R. N. and Blattner, W. A.: Decreased helper T- lymphocytes in homosexual men. II. Sexual practices. Amer. J. Epidemiol. 121: 637-644, 1985.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE		PROJECT NUMBER	
NOTICE OF INTRAMURAL RESEARCH PROJECT		Z01CP05435-02 LHC	
PERIOD COVERED October 1, 1985 to September 30, 1986			
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Analysis of Hydrocarbon-DNA Adducts in Humans and Relation to Cancer Risk			
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)			
PI:	Curtis C. Harris	Chief	LHC NCI
Others:	Ainsley Weston	Visiting Fellow	LHC NCI
	Glennwood Trivers	Res. Biologist	LHC NCI
	Dean L. Mann	Section Chief	LHC NCI
COOPERATING UNITS (if any) University of Oulu, Finland (K. Vahakangas); Louisiana State University, Baton Rouge, LA (M.J. Newman).			
LAB/BRANCH Laboratory of Human Carcinogenesis			
SECTION Biochemical Epidemiology Section			
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892			
TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0.0	
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews			
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Polycyclic aromatic hydrocarbon (PAH)-diol-epoxide-DNA and -protein adducts have been prepared synthetically and subjected to analysis using spectrophotometric, spectrophotofluorimetric and immunological methods. In addition some of the PAH-DNA adducts have been used as both solid phase and fluid phase antigens in competitive and noncompetitive enzyme linked immunosorbant assays (ELISA) to identify human anti-PAH-DNA antibodies in normal healthy individuals.</p> <p>A series of validation studies for the detection of adducts by synchronous fluorescence spectroscopy (SFS) and an ultrasensitive enzyme-linked radioimmunoassay (USERIA) have shown that these methods are corroborative for model adducts studied in controlled systems. However, when applied to the detection of PAH-DNA adducts in human peripheral blood, discrepancies are observed that may be related to the presence in these samples of complex mixtures of adducts. The presence of multiple adduct types in biological macromolecules presents a major problem for both qualitative and quantitative analysis of human biological samples. Analysis of a synthetic mixture containing 5-PAH-DNA adducts has been shown to be possible using SFS, HPLC and computer-based spectral analysis. A method for the extraction of PAH-hemoglobin adduct hydrolysis products has also been developed in order to improve the sensitivity of this method by boosting the total adduct pool.</p>			

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

C. Harris	Chief	LHC	NCI
A. Weston	Visiting Fellow	LHC	NCI
G. Trivers	Res. Biologist	LHC	NCI
D. Mann	Chief, Biochem. Epidemiol. Section	LHC	NCI

Objectives:

1. To continue to identify and characterize PAH-DNA and PAH-protein adducts that may be detected in humans as a result of exposure to PAH contamination of the environment.
2. To utilize the method that has been developed for the detection of BP-hemoglobin adducts for the analysis of human peripheral blood samples.
3. To extend the present level of sophistication of validation experiments in order to determine the relevance of hemoglobin adduct formation. This will be approached by treating rodents with mixtures of PAHs; the metabolic fate of some of these PAHs will be monitored by the use of radiolabeling and all will be monitored by SFS.

Methods Employed:

Formation of PAH-diol-epoxide-DNA adducts was performed by dissolving the reactive hydrocarbon in either DMSO or THF:ethanol (1:24) and mixing with an aqueous solution of DNA in the ratio 1:10 (w/w). Fluorescence characterization of five PAH-DNA adducts and products of acid hydrolysis (NHCl, 90°C, 3 hr) was performed using a Perkin Elmer MFP-66 spectrophotofluorimeter interfaced with a Perkin Elmer series 700 data station.

PAH-hemoglobin adducts are not fluorescent, but a facile method has been developed for the preparation of the major hydrolysis product that is released when a covalent complex of BPDE-hemoglobin is treated with hot (80°C) HCl (0.2 M) for 2-3 hours. Starting with whole human peripheral blood, erythrocytes were packed (and for the purposes of method development, treated with carbon-14-labeled-BPDE), the cells were washed twice in PBS and lysed in hypotonic saline. The cell debris was removed by centrifugation and the hemoglobin was recovered from the supernatant. The protein solution was extracted eight times with diethyl ether and twice with isoamyl alcohol to remove noncovalently bound radioactive material. At this point (i.e., after hydrolysis) the presence in the mixture of hemoglobin and its acid hydrolysis totally quench the fluorescence signal. Therefore, following treatment with HCl (0.2 M, 80°C, 2-3 hr) the hydrolyzed hemoglobin solution was extracted twice with an equal volume of isoamyl alcohol. The two extracts were pooled, evaporated to dryness and redissolved in water for fluorimetric assay.

HPLC, using a reverse-phase C-18 ODS column (4.6 x 250 mm) that was eluted with a linear gradient (30-40%) of methanol in water, was used to separate the

major products that were formed when a mixture of benzo(k)fluoranthene-diol-epoxide-DNA, chrysene-diol-epoxide-DNA, benz(a)anthracene-diol-epoxide-DNA, dibenz(a,c)anthracene-diol-epoxide-DNA and BPDE-DNA adducts were hydrolyzed with HCl (0.1 M, 90°C, 3 hr).

Major Findings:

In a series of validation experiments it was shown for BP-DNA adducts formed in mouse skin that levels of PAH-DNA adducts were detected in the range 0.1 - 1.2 fmol BP/ μ g DNA by both SFS and USERIA. In a second set of assays using DNA isolated from the peripheral blood lymphocytes of coke oven workers it was found that levels of PAH-DNA adducts determined by SFS and USERIA in positive samples were similarly ranked by the two methods, but the USERIA analysis gave two- to fivefold higher estimates of adduct levels.

The analysis of a synthetic mixture of PAH-DNA adducts by HPLC, SFS and computer based (NIH's 'M' Lab System) contour mapping of three dimensional SFS, has permitted the characterization of the optimal synchronous fluorescence yield for the fluoranthene chromophore (293 nm using a synchronous wavelength difference or DL of 178 nm), the phenanthrene chromophore (300 nm, DL 56 nm), the triphenylene chromophore (282 nm, DL 74 nm) and the anthracene chromophore (342 nm, DL 170 nm). The suitability of these types of compounds for these studies was also indicated by this analysis. Unlike the pyrene chromophore, which was previously reported to have a single strong SFS at 379 nm with a DL of 34 nm (BPDE-DNA adducts, therefore, being an excellent choice of model compound), the fluoranthene chromophore has a very broad emission spectrum and several minor peaks are evident in the SFS.

Synthetic modification of hemoglobin with ^{14}C -labeled BPDE was assayed, but only low levels of modification (approximately 20 fmol BPDE/ μ g protein) were achieved. Globin was modified with ^3H -labeled BPDE to a greater extent (17 pmol BPDE/micro, gm protein). It was possible to consistently recover 60% of a sample of 500 fmol BPDE that was covalently bound to 5 mg hemoglobin using acid hydrolysis (20 experiments SD \pm 2.9%) and isoamyl alcohol extraction of the PAH-tetrol products. These materials were also examined by fluorimetry and found to be identical to previously identified pyrene-like compounds.

Some human serum samples were found to contain antibodies that showed significant levels of reactivity with the synthetically prepared PAH-DNA adducts (ChDE-DNA, BADE-DNA and BPDE-DNA) in noncompetitive ELISAs. Subsequent testing of these samples indicated that some of the antibodies reacted with epitopes shared by more than one of these antigens but that others appeared to react with adduct-specific epitopes.

Publications:

Astrup, H., Wakhisi, J., Vahakangas, K., Wasunna, O. A. and Harris, C. C.: Detection of 8,9-dihydro-(7¹guanyl)-9-hydroxyafatoxin B₁ in human urine. Environ. Health Perspect. 62: 105-109, 1985.

Harris, C. C. (Ed.): Biochemical and Molecular Epidemiology of Cancer. New York, A. L. Liss. (In Press)

Harris, C. C.: Future directions for use of carcinogen-DNA adducts as internal dosimeters to monitor human exposure to mutagens and carcinogens. Environ. Health Perspect. 62: 185-192, 1985.

Harris, C. C., Vahakangas, K., Newman, M. J., Trivers, G. E., Shamsuddin, A., Sinopoli, N., Mann, D. L. and Wright, W. E.: Detection of benzo(a)pyrene diol-epoxide-DNA adducts in peripheral blood lymphocytes and antibodies to the adducts in serum from coke oven workers. Proc. Natl. Acad. Sci. USA 82: 6672-6676, 1985.

Haugen, A., Becher, G., Benestad, C., Vahakangas, K., Trivers, G. E., Newman, M. J. and Harris, C. C.: Biomonitoring of individuals exposed to high levels of PAH in the work environment. In Cooke, W. M. and Dennis, A. G. (Eds.): Proceedings for the Tenth International carbons. Columbus, Ohio, Battelle Press. (In Press)

Haugen, A., Becher, G., Beneskad, C., Vahakangas, K., Trivers, G. E., Newman, M. J. and Harris, C. C.: Determination of polycyclic aromatic hydrocarbons (PAH) in the urine, benzo(a)diol epoxide-DNA adducts in lymphocyte DNA and antibodies to the adducts in sera from coke oven workers exposed to measured amounts of PAH in the work atmosphere. Cancer Res. (In Press)

Hoffmann, D. and Harris, C. C. (Ed.): Mechanisms of Tobacco Carcinogenesis. Cold Spring Harbor, Banbury Report 24, Cold Spring Harbor Laboratory. (In Press)

Vahakangas, K., Haugen, A. and Harris, C. C.: An applied synchronous fluorescence spectrophotometric assay to study benzo(a)pyrene-diol-epoxide-DNA adducts. Carcinogenesis 6: 1109-1116, 1985.

Vahakangas, K., Trivers, G., Rowe, M. and Harris, C. C.: Benzo(a)pyrene diol epoxide-DNA adducts detected by synchronous fluorescence spectrophotometry. Environ. Health Perspect. 62: 101-104, 1985.

Weston, A., Trivers, G. E., Vahakangas, K., Newman, M. J.: Detection of carcinogen-DNA adducts in human cells and antibodies to these adducts in human sera. Prog. Exp. Tumor Res. (In Press)

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05477-01 LHC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Activation of Oncogenes by Ultraviolet Light		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: Douglas E. Brash Sr. Staff Fellow LHC NCI Others: K. Kraemer Res. Scientist LMC NCI		
COOPERATING UNITS (if any) Department of Dermatology, Massachusetts General Hospital, Boston, MA (H. Baden, T.B. Fitzpatrick).		
LAB/BRANCH Laboratory of Human Carcinogenesis		
SECTION Carcinogen Macromolecular Interaction Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 0.5	PROFESSIONAL: 0.5	OTHER: 0.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> We transfected a UV-irradiated shuttle vector carrying a tRNA gene into normal and xeroderma pigmentosum complementation group A (excision repair defective) cells, and measured mutations and UV-induced lesions at each base pair in the gene. Four mutation hotspots were found in XPA; two were photoreactivatable, implicating cyclobutane dimers and two were nonphotoreactivatable, implicating a nondimer lesion. The dimer mutation sites were not found in normal cells, implying that in normal cells dimers were not mutagenic because they were excised. A less-readily excised nondimer lesion does generate mutations in normal cells. The lesion frequency was not correlated with mutation frequency at these hotspots; preferential excision and unidentified lesions were excluded as explanations. Mutation hotspots thus occur at particular spots in the sequence independent of lesion frequency. Such a spot would occur if lesions blocked DNA replication, reducing the number of mutants recovered, except at the mutation hotspots. </p> <p> To examine the origin of human skin cancer, we examined UV photoproducts in the cloned Ki-ras gene, which is reported to be activated in UV-induced mouse skin tumors. A DNA damage hotspot is predicted in codon 61 of Ki-ras and N-ras, but not Ha-ras nor at codon 12. The lack of hotspot in Ha-12, Ha-61, and Ki-12 has been found; Ki-61 is being examined. Concurrently, we are transfecting UV-irradiated cloned c-Ha-ras and the c-Ki-ras minigene into NIH 3T3 cells to locate sites of activation. We have also isolated genomic DNA from human basal and squamous cell carcinomas; these will be screened for activated ras oncogenes with oligonucleotide probes and by 3T3 transfection. </p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project

Douglas Brash	Sr. Staff Fellow	LHC	NCI
Kenneth Kraemer	Res. Scientist	LMC	NCI

Objectives:

To determine the mechanism of UV mutation and the identity of the UV mutagenic lesion. To determine the nature of the xeroderma pigmentosum excision repair defect. To determine whether activation of particular oncogenes is determined by DNA damage hotspots. To identify oncogenes activated in human skin tumors.

Methods Employed:

The shuttle vector is transfected into human cells using DEAE-dextran, allowed to replicate, the DNA isolated and incubated with Dpn I to digest unreplicated plasmid, and transformed into *E. coli*. Lac⁻ mutant colonies are picked, the vector DNA isolated, and the sequence determined by supercoil sequencing. Lesion frequencies are measured by end-labeling vector DNA, incubated with dimer-specific endonuclease to cut the phosphodiester bond at cyclobutane dimers or with hot piperidine to break at (6-4) photoproducts, and analyzed on DNA sequencing gels. Cyclobutane dimers were photoreactivated in vitro with purified *E. coli* photoreactivating enzyme. Transfection of NIH 3T3 cells using the calcium phosphate technique. Genomic DNA is isolated from human tumors by crushing in liquid nitrogen, homogenizing, digesting with proteinase K and SDS, centrifuging on CsCl gradients, and dialyzing.

Major Findings:

1. UV-induced mutation hotspots in normal human cells are due to a nondimer lesion. In the dimer-excision defective xeroderma pigmentosum cell (complementation group A), two new hotspots appeared which were photoreactivatable, i.e., due to cyclobutane dimers.
2. The mutation frequency was not correlated to the frequency of either dimers or 6-4 photoproducts. Since this lack of correlation was found in XPA, preferential excision repair at sites with low mutation frequencies is not the explanation. Since at two sites the lesion was identified as the cyclobutane dimer, the results are not explained by hypothesizing a third lesion. We are led to conclude that some sites in the DNA sequence are predisposed to mutate rather than block DNA replication. This suggestion is supported by the fact that three spontaneous mutation hotspots are also UV mutation hotspots.
3. c-Ki-ras, reported to be activated in UV-induced mouse skin tumors, is predicted to have a DNA damage hotspot at codon 61, but not 12, based on rules we previously discovered for predicting UV photoproduct frequency from DNA sequence. Ha-ras should not have a hotspot at either site. We have measured dimers and 6-4 photoproducts at the latter three sites and find the result

predicted. Codon 61 of K-ras is being measured. This result offers the potential to explain why different carcinogens activate different oncogenes even in the same cell type and predicts that if human skin tumors contain ras oncogenes activated by UV, activation will occur at Ki-61 rather than Ha.

Publications:

None

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05478-01 LHC																								
PERIOD COVERED October 1, 1985 to September 30, 1986																										
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Ha-ras Transformed NHBE Cells: A Model for Metastasis																										
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 30%;">PI: G. H. Yoakum</td> <td style="width: 30%;">Senior Staff Fellow</td> <td style="width: 20%;">LHC</td> <td style="width: 20%;">NCI</td> </tr> <tr> <td colspan="4" style="padding-top: 10px;">Others: C. C. Harris</td> </tr> <tr> <td></td> <td>Chief</td> <td>LHC</td> <td>NCI</td> </tr> <tr> <td></td> <td>Lance Liotta</td> <td>LP</td> <td>NCI</td> </tr> <tr> <td></td> <td>D. C. Rao</td> <td>LP</td> <td>NCI</td> </tr> <tr> <td></td> <td>Visiting Fellow</td> <td></td> <td></td> </tr> </table>			PI: G. H. Yoakum	Senior Staff Fellow	LHC	NCI	Others: C. C. Harris					Chief	LHC	NCI		Lance Liotta	LP	NCI		D. C. Rao	LP	NCI		Visiting Fellow		
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COOPERATING UNITS (if any) Laboratory of Developmental Biology and Anomalies, NIDR, Bethesda, MD (E. Schiffman)																										
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SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> An in vitro model was developed to study the multistage progression in malignancy of human bronchial epithelial cells that were transformed to immortal cell lines with measurable malignant potential following transfection with Harvey <u>ras</u> oncogene (v-Ha-<u>ras</u>). Progressively malignant cell lines derived from this transformation were selected by continued growth in tissue culture (TBE-1), anchorage-independent growth in soft agar (TBE-1SA), and xenogenic transfer of TBE-series tumor tissues between mice. The TBE-1SA cell line has a shorter average latency period for subcutaneous primary tumors in athymic nude mice, higher frequency of successful transplantation, and more frequent metastasis to the liver, spleen, and lungs from primary tumors than tumorigenic cell lines selected for progression by continued growth in cell culture. The secondary growth of tumors that were passaged between mice also led to increased malignancy for each type of TBE-cell line tested. Tumors from TBE cell lines have a similar array of histologic morphologies, and decreased expression of histocompatibility markers (HLA class I) were less prevalently expressed by tumor cells than those cell lines that were most tumorigenic. The karyotypic variation and complex pattern of surface markers observed in different TBE cell lines and tumor tissue is consistent with an increasing adaptive change that alters the expression of multiple genes during malignant progression. Mechanistically, this is consistent with increased adaptation by cell populations derived from v-Ha-<u>ras</u> transformed human epithelial cells that progress to malignancy and metastasis. </p>																										

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

George H. Yoakum	Senior Staff Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
Lance Liotta	Chief	LP	NCI
D. C. Rao	Visiting Fellow	LP	NCI

Objectives:

To develop an in vitro model of metastasis utilizing the highly malignant variant cell lines selected from v-Ha-ras transformed human bronchial epithelial cell lines. These studies will provide insight about the biochemical and genetic changes that occur during the multistage process of development toward complete malignancy by the v-Ha-ras transformed human epithelial cells.

Methods Employed:

i) In vitro tissue culture growth of transformed human bronchial epithelial cells; ii) tumor growth in athymic nude mice; iii) xenogenic transfer of tumors; iv) selection of metastatic phenotypes from animal experiments; v) biochemical analysis of tumor tissue and cell lines recovered by in vitro culture of tumor tissues.

Major Findings:

The tumorigenic potential of v-Ha-ras transformed NHBE cells (TBE-series) provides a unique opportunity to develop model animal systems for the study of human malignancy in vitro. This system has provided the following information about the biological, biochemical, and genetic aspects of multi-stage development of Ha-ras related tumors: i) The v-Ha-ras transformed human bronchial epithelial cells are nontumorigenic immediately after transformation and progress to malignancy by continued growth in cell culture or selection of anchorage-independent subpopulations. ii) The tumorigenic phenotype initially requires long latency periods for expression (9-12 months). iii) The tumors passaged by xenograft between test animals develop select progressive populations of tumor cells with a shorter latency period (35-60 days). iv) The metastatic potential of Ha-ras-transformed TBE cells increases concomitant with tumorigenicity.

Publications:

Harris, C. C., Yoakum, G. H., Lechner, J. F., Willey, J. C., Masui, T., Gerwin, B., Schlegel, S. and Mark, G.: Growth, differentiation and neoplastic transformation of human bronchial epithelial cells. In Harris, C. C. (Ed.): Biochemical and Molecular Epidemiology of Cancer. New York, A. L. Liss. (In Press)

Yoakum, G. H., Lechner, J. F., Gabrielson, E. W., Korba, B. E., Malan-Shibley, L., Willey, J. C., Valerio, M. G., Shamsuddin, A. M., Trump, B. F., and Harris, C. C.: Transformation of human bronchial epithelial cells transfected by Harvey ras oncogene. Science 227: 1174-1179, 1985.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05479-01 LHC																								
PERIOD COVERED October 1, 1985 to September 30, 1986																										
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Detection of Carcinogen DNA Adducts by 32P Postlabeling																										
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: Vincent L. Wilson</td> <td style="width: 33%;">Sr. Staff Fellow</td> <td style="width: 17%;">LHC</td> <td style="width: 17%;">NCI</td> </tr> <tr> <td colspan="4" style="padding-top: 10px;">Others:</td> </tr> <tr> <td>Simon M. Plummer</td> <td>Visiting Fellow</td> <td>LHC</td> <td>NCI</td> </tr> <tr> <td>Philip Smith</td> <td>Hall-Shields Fellow</td> <td>LHC</td> <td>NCI</td> </tr> <tr> <td>Dean Mann</td> <td>Section Chief</td> <td>LHC</td> <td>NCI</td> </tr> <tr> <td>Curtis C. Harris</td> <td>Chief</td> <td>LHC</td> <td>NCI</td> </tr> </table>			PI: Vincent L. Wilson	Sr. Staff Fellow	LHC	NCI	Others:				Simon M. Plummer	Visiting Fellow	LHC	NCI	Philip Smith	Hall-Shields Fellow	LHC	NCI	Dean Mann	Section Chief	LHC	NCI	Curtis C. Harris	Chief	LHC	NCI
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Curtis C. Harris	Chief	LHC	NCI																							
COOPERATING UNITS (if any) Department of Nutrition and Food Science, Massachusetts Institute of Technology, Boston, MA (J.M. Essigman); Department of Toxicology, Karolinska Institute, Stockholm, Sweden (R.C. Grafstrom).																										
LAB/BRANCH Laboratory of Human Carcinogenesis																										
SECTION Biochemical Epidemiology Section																										
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892																										
TOTAL MAN-YEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 1.0																								
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SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) The ability to detect low levels of carcinogen DNA adducts in the tissues of people environmentally exposed to chemical carcinogens is invaluable to epidemiological studies of the incidence of cancer in selective populations. A number of selective methodologies have been developed to quantitate carcinogen DNA adducts. The Randerath 32P postlabeling technique provides a fingerprint analysis of only polycyclic aromatic hydrocarbon type DNA adducts. However, the 32P postlabeling method has been adapted in the present study to enable the detection of small alkylation type carcinogen DNA adducts. The detection and quantitation of O6-MeGua adducts in DNA has been shown, by the use of standards, to be accurate as low as one adduct in at least 1,000,000 guanine residues. The presence of unidentified 32P labeled spots has also been observed from the analysis of DNA from cells treated with acrolein and DNA treated with fecapentaene.																										

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Vincent L. Wilson	Sr. Staff Fellow	LHC	NCI
Simon M. Plummer	Visiting Fellow	LHC	NCI
Philip Smith	Hall Shields Fellow	LHC	NCI
Dean Mann	Chief, Biochem. Epidemiology Sect.	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

To provide a sensitive physical technique for the quantitation of small DNA adducts and selective forms of base damage in DNA from people environmentally exposed to carcinogens. To establish the usefulness of the analysis of O⁶-MeGua and other selective adducts to the study of the relationship of carcinogen exposure to human cancer.

Methods Employed:

DNA treated with N-methyl-N-nitrosourea (MNU) or other selective direct acting carcinogen, liver DNA from NMU-treated rodents, and human tissue DNA from unknown environmental exposure to methylating carcinogens, will be enzymatically digested to 3'-monophosphate nucleotides. The normal and adducted nucleotides will be separated by HPLC and then labeled and quantitated by the newly adapted 32P postlabeling method (Wilson et al., Anal. Biochem. 152:275-284, 1986). The 3'-monophosphate adducted nucleotide standards must be synthesized for each alkylation product quantitated.

Major Findings:

This is a new project developed following the successful adaptation of the Randerath 32P postlabeling methodology to the study of the normal and small molecule alkylated nucleotides in nucleic acids. The first alkylation product under study has been O⁶-MEGua. Quantitation of standard mixtures of O⁶-MeGua and guanine by HPLC and subsequent 32P postlabeling has demonstrated the accuracy of the methodology to one adduct in 1,000,000 guanine residues. The limit of detection is presently being determined.

The usefulness of this methodology for the identification of other alkylation (and DNA damage) products of exposure to environmentally important contaminants is under study. DNA isolated from acrolein-treated human fibroblasts contains several unidentified adducted or damaged nucleotides not seen previously by other techniques. Fecapentaene treated DNA contains at least four unknown products which may be separated and possibly identified by subsequent LC/MS analysis.

Publications:

None

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05480-01 LHC																												
PERIOD COVERED October 1, 1985 - September 30, 1986																														
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Genetic Polymorphisms and Relationship to Cancers of the Respiratory Tract																														
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 40%;">PI: Curtis C. Harris</td> <td style="width: 20%;">Chief</td> <td style="width: 20%;">LHC</td> <td style="width: 20%;">NCI</td> </tr> <tr> <td>Others: Ainsley Weston</td> <td>Visiting Fellow</td> <td>LHC</td> <td>NCI</td> </tr> <tr> <td>James C. Willey</td> <td>Biotech Training Fellow</td> <td>LHC</td> <td>NCI</td> </tr> <tr> <td>Susan P. Banks-Schlegel</td> <td>Sr. Staff Fellow</td> <td>LHC</td> <td>NCI</td> </tr> <tr> <td>George C. Yoakum</td> <td>Sr. Staff Fellow</td> <td>LHC</td> <td>NCI</td> </tr> <tr> <td>Brenda I. Gerwin</td> <td>Research Chemist</td> <td>LHC</td> <td>NCI</td> </tr> <tr> <td>Dean L. Mann</td> <td>Section Chief</td> <td>LHC</td> <td>NCI</td> </tr> </table>			PI: Curtis C. Harris	Chief	LHC	NCI	Others: Ainsley Weston	Visiting Fellow	LHC	NCI	James C. Willey	Biotech Training Fellow	LHC	NCI	Susan P. Banks-Schlegel	Sr. Staff Fellow	LHC	NCI	George C. Yoakum	Sr. Staff Fellow	LHC	NCI	Brenda I. Gerwin	Research Chemist	LHC	NCI	Dean L. Mann	Section Chief	LHC	NCI
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COOPERATING UNITS (if any) New England Medical Center, Boston, MA (T. Krontiris); Children's Hospital of LA, Los Angeles, CA (W. Benedict); National Institute of Occupational Health, Oslo, Norway (A. Haugen)																														
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SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>There is a clear association between smoking and lung cancer, but it is still not known why some individuals, who are heavily exposed to large concentrations of chemical carcinogens, do not develop tumors, whereas others do. These observations provide circumstantial evidence for the involvement of a genetic factor that predisposes for tumor formation. Recent restriction fragment length polymorphism (RFLP) studies have shown that the loss of normal cellular sequences from chromosome 13 (in the case of retinoblastoma) and chromosome 11 (in the cases of Wilm's tumor and bladder cancer) have been associated with malignancy. It appears that this method might be generally applied to the analysis of inherited susceptibility to cancer and therefore be informative in risk assessment for lung cancer. Tumor and normal tissue from high molecular weight DNA samples have been collected from more than 30 cancer patients for restriction enzyme digestion and Southern analysis. Initial experiments have centered on examination of genes located on the short-arm of chromosome 11; loss of allelic fragments during tumorigenesis have been detected in 15% of samples at the cellular Harvey <u>ras</u> locus and in 13% of samples at the insulin locus. Experiments that examine more than 20 additional genetic loci throughout the human genome for these DNA samples are in progress.</p>																														

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

C. Harris	Chief	LHC	NCI
A. Weston	Visiting Fellow	LHC	NCI
J. Willey	Biotech Training Fellow	LHC	NCI
G. Yoakum	Sr. Staff Fellow	LHC	NCI
B. Gerwin	Research Chemist	LHC	NCI
D. Mann	Chief, Biochem. Epidemiology Sect.	LHC	NCI

Objectives:

To examine the DNA restriction patterns of normal and lung tumor tissues with human genomic and cDNA probes. Initial studies will focus on genetic loci assigned to the short (p) arm of chromosome 11 since deletions in this region of the genome have been shown to be associated with Wilm's tumor and other malignant conditions of the genitourinary tract. Known genetic loci throughout the human genome will also be examined to determine whether specific genetic polymorphisms are associated with carcinogenesis per se or whether deletion of genetic loci in carcinogenesis is part of a more general mechanism.

Methods Employed:

High molecular weight DNA was extracted from fresh or frozen tissues by gentle mechanical disruption of the tissue; enzyme digestion to degrade protein and RNA; phenol, phenol:chloroform extractions; alcohol precipitation and spooling high molecular weight materials.

Restriction analysis was performed by the method of Southern. DNA was digested to completion with appropriate restriction enzymes, phenol extracted and subjected to agarose gel electrophoresis. The DNA was transferred (by Southern blotting) to nitrocellulose filters that were baked to immobilize the DNA. The filters were hybridized to cloned human genomic or cDNA fragments of known genetic loci, which were previously radiolabeled to a very high specific activity with phosphorous 32 (according to the primer method of Vogelstein (Anal. Biochem. 137: 266, 1984)). X-ray films were exposed to the filters in light-proof cassettes at -70°C for periods of between 4 hrs and 6 days.

Major Findings:

Three genetic loci found on the short-arm of chromosome 11 have been examined. Only data pertaining to patients that are heterozygous for a given polymorphic genetic locus are informative since a genetic deletion resulting in hemizyosity is being monitored. Examination of the c-Ha-ras locus using a BamHI digest in 24 human lung tumors revealed allelic deletions in 2/13 heterozygotes when compared to matched normal tissue samples. Examination of the calcitonin locus using a TaqI digest in eight human lung tumors revealed allelic deletions

in 0/4 heterozygotes. Examination of the 5' tandemly repeated (VTR) region of the insulin gene locus using a PvuII digest in 31 human lung tumors revealed allelic deletions in 3/22 heterozygotes.

With respect to the insulin VTR region, the tumor of one of the homozygous patients contained an extra piece of DNA (possibly as the result of a duplication or point mutation), which gave rise to an abnormally large allele. In addition one of the heterozygous patients, whose tumors showed identical restriction patterns to the normal tissue, appeared to have one abnormally large allelic fragment. Preliminary data suggest that this previously unobserved allele is the result of an increase in the number of short (<100 base pairs) repeating units rather than a restriction site mutation.

Publications:

None

Patents:

None

ANNUAL REPORT OF
THE LABORATORY OF MOLECULAR CARCINOGENESIS
CHEMICAL AND PHYSICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1985 to September 30, 1986

The Laboratory of Molecular Carcinogenesis (LMC) plans, develops, and conducts a research program designed to (1) clarify the molecular biology of carcinogenesis; (2) elucidate the fundamental nature of the interaction of carcinogenic agents, especially chemical, with biological systems in the induction of cancer; (3) identify those environmental and endogenous factors which relate to and modify the carcinogenic process; and (4) clarify the metabolic regulatory processes which are related to carcinogenesis.

The goal of the Laboratory of Molecular Carcinogenesis is to understand the molecular basis of carcinogenesis with the view toward identifying susceptible populations and preventing human cancer. The research program is designed to understand the molecular basis by which carcinogenic agents cause malignant transformation and to identify and characterize those exogenous and endogenous factors involved in carcinogenesis. The Laboratory seeks to clarify the metabolic interaction of exogenous and endogenous agents in the living organism at the molecular, cellular and organism levels and seeks to understand the consequences of these interactions in terms of cell regulation and carcinogenesis. The processes are studied in biological preparations and cells from experimental animals and humans.

The course of the Laboratory research program has been markedly affected by the powerful new techniques of molecular biology and immunology. Several of our staff are highly experienced in DNA recombinant and related techniques, protein chemistry, and hybridoma technology. The power and precision of these techniques have had a highly positive influence on the progress of many of the projects of the Laboratory.

Metabolic Control Section - Studies (1) the metabolic activation and detoxification of the polycyclic hydrocarbons (PCH) and other carcinogens and drugs and the relationship of this metabolism to individual sensitivity and susceptibility to carcinogenesis; (2) regulation and structure of the genes for the enzymatic system primarily responsible for the metabolic activation and detoxification of PCH and other chemical carcinogens.

This section studies the molecular events of malignant transformation induced by chemical carcinogens, mainly those of the PCH class. The aim is to understand the enzymatic conversion of carcinogens to either detoxified forms or to active carcinogenic forms. Higher organisms have systems for the detoxification and elimination of foreign chemical compounds, including carcinogens. These systems primarily involve microsomal cytochrome P-450 mixed-function oxygenases, but also include epoxide hydratase and conjugating enzymes. The

vast majority of foreign compounds are processed by these enzyme systems. The mixed-function oxygenases are influenced by a variety of environmental factors such as drugs, pesticides and carcinogens and are influenced by the nutritional and hormonal state of the animal. The age, sex and genetic makeup also determine enzyme activity. Work in this Laboratory provided the key studies which showed that this enzyme system was responsible for the activation of PCH procarcinogens to their ultimate carcinogenic forms. A primary goal is to define the enzymatic mechanism by which polycyclic hydrocarbons are activated either to carcinogenic forms or to detoxified products. As these enzymes are characterized and as sensitive methods are developed for their assay, it may be possible to characterize an individual's enzymatic makeup with respect to carcinogen metabolism and to understand the relationship between this metabolism and individual susceptibility to PCH-induced carcinogenesis.

The approach is to identify and fully characterize the enzymes responsible for carcinogen activation and metabolism. In addition, we seek to understand the molecular biology and regulation of this system both at the genetic and epigenetic levels. We plan to assess the types and amounts of these enzymes in human populations using molecular biological, immunological and metabolic approaches. We will carry out multileveled investigations of the carcinogen metabolizing enzyme systems, continuing our use of HPLC to study carcinogen metabolites, using monoclonal antibodies (MAb) to study the properties of the enzymes and using recombinant DNA and other molecular biological techniques to study the structure and regulation of the genes for the enzymes of carcinogen-metabolizing systems.

MAbs have been prepared to cytochromes P-450 of rats treated with phenobarbital, 3-methylcholanthrene, pregnenolone 16- α -carbonitrile and ethanol, and an environmentally induced cytochrome P-450 in fish (scup). These MAbs are characterized for their epitope specificity toward different cytochrome P-450 isozymes. A MAb to environmentally induced fish cytochrome P-450 cross-reacts with 3-methylcholanthrene-inducible rat cytochrome P-450 and may be useful for monitoring polluted marine environments. MAbs to an ethanol-inducible and nitrosamine metabolizing cytochrome P-450 (P-450et) have been utilized for identification of P-450et in liver microsomal preparations of rats which were untreated or treated with different chemical agents. MAbs to pregnenolone 16- α -carbonitrile- and ethanol-inducible rat cytochromes P-450 detect cytochrome P-450 isozymes in human liver. MAbs to human cytochromes P-450 from placenta, mitochondria and liver were prepared.

MAbs to cytochromes P-450 have been used as specific probes for epitope-specific cytochromes P-450 in human liver samples obtained from different individuals. Western blot analysis with antisera or a MAb to rat ethanol-induced P-450 detected a P-450 in human liver homogenates and microsomes. The level of the immunodetectable P-450 varied among livers from different individuals. A human liver P-450 was immunopurified using the MAb and was structurally characterized. We are additionally developing a radioimmunoassay for human cytochrome P-450 as a rapid, efficient method for screening large numbers of samples from human tissues.

3-methylcholanthrene (MC) inducible P-450s have been immunopurified from the livers of rats, C57Bl/6 and DBA/2 mice, guinea pigs and hamsters. Their primary structures were compared by amino acid sequence analysis and peptide mapping and revealed varying degrees of homology. Several previously unidentified amino terminal residues of an immunopurified mouse P-450 were identified, and the sequence of a guinea pig liver P-450 was determined for the first time. A rat lung P-450 was also immunopurified and was indistinguishable from a liver P-450 by several criteria. Using a MAb to ethanol-inducible rat liver P-450, a P-450 has been purified from human liver and was structurally characterized. Since immunopurification typically denatured the P-450s, an alternative approach involving antigen-exchange was developed in which inactive denatured P-450 displaces active P-450 from the immunoabsorbent. Such epitope-specific exchange may be generally applicable to preparation of proteins with retention of activity. The epitopic structure of the P-450 surface was mapped by evaluating the binding of MAbs to various P-450 peptide fragments and by computer-aided alignment of homologous P-450s which are epitopically related. Examination of the P-450 dependent testosterone metabolism in 3- and 24-month old rats indicated that the hydroxylation patterns as well as content of liver constitutive P-450s varied with age.

Several forms of P-450 were purified from rat liver and kidney, and rabbit liver microsomes. These purified P-450s were characterized catalytically, structurally and spectrally, and classified on the basis of immunological cross-reactivity. Several forms of P-450 were used to study the conformation of the protein surface, using monoclonal antibodies (MAbs) to specific cytochromes P-450. MAbs were also used for immunopurification of P-450 with catalytic activity, for making a P-450 bioreactor and for evaluating epitope topography of the microsomal membrane.

Certain P-450s are developmentally regulated. These include testosterone-16 α -hydroxylase, an adult male specific P-450, and testosterone-15 α -hydroxylase, an adult female specific P-450. These two enzymes increase during rat development and their increase is mediated through blood androgen levels and pulsatile levels of growth hormone. We have been analyzing two classes of P-450s: one class of P-450 including P-450f and P-450PB-1 have no known steroid hydroxylase activities. These P-450s increase during development in both males and females and reach maximal levels at rat maturity. This increase is independent of androgens. We cloned and sequenced cDNAs for these enzymes and found that they share 75% amino acid homology. The cDNA probes were used to show that these P450s increase during development as a result of transcriptional activation of their respective genes. These P450s are probably linked and coordinately regulated.

The cDNA of a P-450 that is induced by steroids was cloned and sequenced. This P450, designated P-450PCN1 (PCN = pregnenolone-16 α -carbonitrile), was found to be absent in noninduced rats and induced by PCN and phenobarbital. Another P-450 cDNA was isolated based on its sequence homology with P-450PCN1 and designated P-450 PCN2. P-450PCN2 has 90% amino acid sequence similarity with P-450PCN1. This homology is present in three distinct regions of 99% to 100% similarity surrounded by regions of 80% to 85% similarity. These data suggest that gene

conversion events have played a role in evolution of this P-450 family. Interestingly, P-450PCN2 is a developmentally regulated P-450. Its level increases in young male and female rats but declines in female rats at puberty and continues to increase in male rats, reaching maximal levels at maturity.

In contrast to P-450PCN1, P-450PCN2 is not induced by steroids such as PCN and dexamethasone. Similar to P-450PCN1, P-450PCN2 is induced by phenobarbital. Induction and developmental studies suggest that both P-450s possess testosterone-6 β -hydroxylase activity.

We have been studying the enzyme P-450j which has a high activity toward the potent carcinogen N-nitrosodimethylamine. The demethylation reaction carried out by this P-450 leads to highly reactive intermediates that can bind and mutate DNA. A monoclonal antibody against rat P-450j was used to isolate rat and human P-450j cDNA clones. These cDNAs were sequenced and their nucleotide and deduced amino acid sequences were 78% and 80% similar, respectively. The rat P-450j monoclonal antibody and cDNA clones were utilized as probes to determine the mechanism whereby P-450j is regulated by ethanol, acetone, and the alcohol dehydrogenase inhibitors, 7-methyl-pyrazole and pyrazole. A unique translational control of P-450j by these compounds was uncovered. The fourfold elevation in P-450j protein in rat microsomes by these agents is not accompanied by an increase in its mRNA. In contrast to this post-transcriptional regulation, P-450j is increased during the first few days after birth through a massive increase in gene transcription rate.

Infectious recombinant vaccinia viruses have been constructed containing the full length cDNAs of P1-450 and P3-450. Human and mouse cells infected with the recombinant viruses showed high level expression of the authentic proteins as detected by immunoblotting. The expressed proteins are enzymatically active and exhibit distinguishable substrate specificities. We have also constructed recombinant retroviruses containing the cytochromes P1-450 and P3-450. Cells infected with these recombinants express cytochrome P3-450. Preliminary experiments indicate that the protein is enzymatically active. This system is now amiable to mutation and transformation analysis.

Protein Section - Studies (1) the relationship between chromatin structure and gene expression and (2) mechanisms by which chromosomal proteins affect the structure and function of chromatin.

In the nucleus of the cell, the DNA is stored and packed in discrete nucleoprotein structures. Regulation of the information encoded in DNA is dependent on specific protein-nucleic interactions. The main aim of the Protein Section is to clarify the protein-nucleic acid interactions which affect the structure and regulate the function of chromatin, chromosomes and specific genes. This broad goal is achieved by isolating specific chromosomal proteins, developing immunochemical assays for these proteins and immunocytochemical techniques to study the organization of specific chromosomal proteins in chromatin and chromosomes. An additional avenue of investigation involves in vitro studies on the binding of specific chromosomal proteins to various DNA structures. Genetic engineering approaches are a powerful and versatile approach to elucidate the

cellular role of proteins. Therefore, genes for chromosomal proteins are isolated and characterized. The relation between the chromatin structure and gene expression of genes coding for cytochrome P-450 is investigated in detail.

In the past year, we have produced antibodies against nonhistone chromosomal proteins and developed immunoaffinity chromatographic techniques which allow the isolation of nucleoprotein complexes enriched in defined chromosomal proteins. We have elucidated the mechanisms by which certain nonhistone chromosomal proteins bind to DNA as well as isolated a cDNA clone coding for a nonhistone chromosomal protein.

The role of chromosomal proteins in maintaining the structure and regulating the function of chromatin and chromosomes is being studied. Specific antibodies were used to construct immunoaffinity columns for fractionating chromatin. Studies on the exchange of proteins during immunofractionation of chromatin revealed that at low ionic strength there was negligible exchange of proteins between nucleosomes. Nucleosomes enriched in HMG-17 or H1o have been isolated by immunoaffinity chromatography and the DNA present in these nucleosomes examined with a variety of genetic probes. The results indicate that chromatin fragments containing DNA sequences with an open reading frame are enriched in HMG-17 while nucleosomes containing DNA sequences coding for inducible proteins are depleted of H1o. We cloned and sequenced cDNA coding for human chromosomal protein HMG-17 and used this cDNA to probe the genomic organization of the gene. In the human genome, there are over 50 gene equivalents for this cDNA suggesting that the protein is encoded by a multigene family. Southern analysis of the DNA from several transformed human cell lines failed to detect any restriction fragment polymorphism in this gene. The cDNA has some unusual characteristics: only 25% of the transcript is translated, the 5' untranslated region is extremely rich in GC residues, while the 3' untranslated region is very rich in AT residues.

The chromatin structure of genes coding for P-450 enzymes is being investigated. Changes in the chromatin structure upon gene activation is examined by comparing the chromatin structure of these genes in nuclei purified from either normal or carcinogen-treated rats. Micrococcal nuclease digestion revealed that, in the liver, genes coding for the 3-methylcholanthrene-inducible P-450c, P-450d, and P-450m enzymes are not organized in the typical 200 base pair nucleosomal conformation. DNase 1 hypersensitive sites were mapped in each of these genes. These sites were not observed in nuclei isolated from the thymus. These results suggest that in the liver the chromatin structure is altered to allow tissue specific gene expression. Gene induction, by carcinogen treatment is associated with a rearrangement of the nuclear location of the P-450c and P-450d genes and with a change in one of the DNase 1 hypersensitive sites present in the P-450c gene. The results suggest that gene induction is associated with detectable changes in the chromatin structure of these genes.

Nucleic Acids Section - Studies (1) the nature of human genetic predisposition to cancer, (2) interaction of chemical and physical carcinogens with nucleic acids and their actions on the functions of DNA, (3) the relationship between defects in repair of cellular DNA and human cancer and (4) chemically produced alteration of DNA and the repair of such alterations.

The study of human cells defective in repairing damaged DNA was extended, with the rationale that DNA repair-deficient cells are more susceptible to the adverse effects of carcinogens (cell killing, mutagenesis, sister chromatid exchange, and malignant transformation) than their repair-proficient counterparts. A group of 19 human tumor and eight SV40-transformed strains, almost totally deficient in the repair of O-6-methylguanine (O-6-MeG, a modified DNA base made by certain methylating agents), was identified earlier in this project. Such strains are called Mer⁻. Transformation by SV40, Rous sarcoma virus, adenovirus, or Epstein-Barr virus produces Mer⁻ strains. Mer⁺ but not Mer⁻ strains contain about 60,000 copies of a 22,000 MW O-6-MeG-DNA methyltransferase (O-6-DMT) that is responsible for repairing O-6-MeG by demethylation. Cell strains having intermediate amounts of O-6-DMT also have intermediate sensitivity to killing by 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) or by 1-(2-chloroethyl)-1-nitrosourea (CNU) and intermediate sensitivity to the induction of sister chromatid exchanges by MNNG. Human interferons alpha and beta inactivate Mer⁻ tumor strains while Mer⁺ human tumor strains are more resistant to such treatment, indicating an association between defective repair of O-6-MeG and sensitivity to interferons. We found O-6-MeG to be lethal to human cells under certain conditions.

Molecular, cellular and clinical abnormalities in patients with xeroderma pigmentosum (XP) and with the dysplastic nevus syndrome (DNS) of hereditary cutaneous melanoma are being studied. We have developed new assays utilizing plasmids as tools to measure DNA repair and mutagenesis at the molecular level. In the XP cells, we showed that one pyrimidine dimer blocks expression of a transfected gene. Nondimer photoproducts also block expression in XP cells. In normal cells, UV pretreatment of a shuttle vector plasmid, pZ189, resulted in appearance of transitions and transversions. Survival of UV-treated pZ189 was reduced in the XP cells and there was a restricted mutagenic spectrum found. In pZ189 replicated in XP cells, 93% of the base substitution mutations were GC to AT transitions ($p < 0.0009$). The major UV photoproduct, the thymine dimer, was only weakly mutagenic. We found that cultured lymphoblastoid cells from familial DNS patients were hypermutable to UV. In a retrospective study of more than 700 XP patients, we have shown that they have a greater than 1000-fold increased risk of developing basal cell or squamous cell carcinoma or melanomas of the skin. The reported median age of first skin cancer was less than 10 years, a 50-year reduction in comparison to the United States general population.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP04496-09 LMC	
PERIOD COVERED October 1, 1985 to September 30, 1986			
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Chromosomal Proteins and Chromatin Function			
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)			
PI:	Michael Bustin	Acting Section Chief	LMC NCI
Others:	David Landsman	Visiting Fellow	LMC NCI
	Thyagarajan Srikantha	Visiting Fellow	LMC NCI
	Nirmolini Soares	Lab. Tech. (Microbiol.)	LMC NCI
COOPERATING UNITS (if any) Chester Beatty Laboratories, England (Dr. Graham Goodwin) Laboratory of Biology, DCE, NCI, Bethesda, MD (Dr. N. Popescu)			
LAB/BRANCH Laboratory of Molecular Carcinogenesis			
SECTION Protein Section			
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892			
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:	
3	2	1	
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews			
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> The role of chromosomal proteins in maintaining the structure and regulating the function of chromatin and chromosomes is being studied. Specific antibodies were used to construct immunoaffinity columns for fractionating chromatin. Studies on the exchange of proteins during immunofractionation of chromatin revealed that at low ionic strength there was negligible exchange of proteins between nucleosomes. Nucleosomes enriched in HMG-17 or H1o have been isolated by immunoaffinity chromatography and the DNA present in these nucleosomes examined with a variety of genetic probes. The results indicate that chromatin fragments containing DNA sequences with an open reading frame are enriched in HMG-17 while nucleosomes containing DNA sequences coding for inducible proteins are depleted of H1o. We cloned and sequenced cDNA coding for human chromosomal protein HMG-17 and used this cDNA to probe the genomic organization of the gene. In the human genome, there are over 50 gene equivalents for this cDNA suggesting that the protein is encoded by a multigene family. Southern analysis of the DNA from several transformed human cell lines failed to detect any restriction fragment polymorphism in this gene. The cDNA has some unusual characteristics: only 25% of the transcript is translated, the 5' untranslated region is extremely rich in GC residues, while the 3' untranslated region is very rich in AT residues. </p>			

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Michael Bustin	Acting Section Chief	LMC	NCI
David Landsman	Visiting Fellow	LMC	NCI
Thyagarajan Srikantha	Visiting Fellow	LMC	NCI
Nirmolini Soares	Lab. Tech. (Microbiol.)	LMC	NCI

Objectives:

To understand the mechanism of gene regulation and its relation to neoplasia by studying the role of defined chromosomal proteins in maintaining the structure and regulating the function of chromatin and chromosomes in normal and transformed cells. Studies are designed to give insights into the chemical nature of chromosomal proteins, their immunological specificities, the manner in which they interact with DNA and the regulation of the expression of genes coding for these proteins.

Methods Employed:

The regulated expression of the genetic information encoded in DNA is dependent on specific protein-nucleic acid interactions. To facilitate the study of specific chromosomal proteins, their interactions with DNA, and their cellular function, we have elicited antibodies against specific proteins and used the antibodies to study the structure and function of chromatin. Proteins are purified from isolated nuclei by differential precipitation, size exclusion chromatography and ion exchange chromatography. Synthetic peptides are prepared by solid phase synthesis. Polyclonal and monoclonal antibodies are elicited in rabbits and mice, respectively. Chromatin is isolated from purified nuclei. The antigenic activity of the purified chromatin and isolated proteins is measured by ELISA, immunoblotting and radioimmune assays. Nucleosomes are prepared by nuclease digestion. Immunoaffinity columns are prepared by the cyanogen bromide procedure using purified Ig. cDNA clones are isolated from expression libraries prepared from the mRNA isolated from transformed human cells. The clones are characterized, propagated in plasmids and the DNA sequence determined. The cDNA clones are used to isolate the human genes coding for the proteins and to study gene expression in various tissues by analyzing the RNA.

Major Findings:

This project led to significant advances in several areas: 1) Immunochemical methods for assaying chromosomal proteins and for investigating chromatin have been developed; 2) Certain aspects of the specificity of the interaction of certain nonhistone proteins with DNA have been clarified; 3) The cDNAs coding for HMG-17 and for HMG-14 nonhistone chromosomal proteins have been isolated and sequenced.

In the past year, we have used immunoaffinity columns to fractionate chromatin and characterize the DNA sequences associated with chromosomal proteins HMG-17

and H1o. The problem of exchange of proteins during chromatin fractionation has been investigated in detail. We found that below 40 mM NaCl there was little exchange of proteins among nucleosomes. Using the appropriate ionic strength condition, we have purified nucleosomes highly enriched in H1o and in HMG-17. Characterization of the DNA sequences associated with these chromosomal proteins, by hybridization with specific probes, revealed that inducible genes, such as genes coding for cytochrome P-450, are depleted of H1o and that DNA sequences coding for proteins are enriched in HMG-17.

The specificity of the interaction between chromosomal proteins HMG-1 and HMG-2 has been investigated using a plasmid which contains DNA sequences that can adopt the Z-DNA conformation and palindromic sequences that can form cruciform structures. We found that there is a significant difference between the binding of the two HMG proteins to this plasmid suggesting that they are involved in distinguishable cellular functions.

A clone containing the entire cDNA coding for human chromosomal protein HMG-17 has been isolated and sequenced. Genomic analysis indicated that this protein is coded by a multigene family. In the human genome there are about 50 gene copies. The isolation of genomic clones coding for this protein is in progress. We have also isolated the cDNA coding for human chromosomal protein HMG-14.

Publications:

Bustin, M. and Soares, N.: Differential binding of chromosomal proteins HMG-1 and HMG-2 to superhelical DNA. Biochem. Biophys. Res. Comm. 133: 633-640, 1985.

Druckmann, S., Mendelson, E., Landsman, D. and Bustin, M.: Immunofractionation of DNA sequences associated with HMG-17 in chromatin. Exp. Cell Res. (In Press).

Landsman, D., Mendelson, E., Druckmann, S. and Bustin, M.: Exchange of proteins during immunofractionation of chromatin. Exp. Cell Res. 163: 95-102, 1985.

Landsman, D., Soares, N., Gonzalez, F. J. and Bustin, M.: Chromosomal protein HMG-17: Complete human cDNA sequence and evidence for a multigene family. J. Biol. Chem. 261: 7479-7484, 1986.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP04517-10 LMC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) DNA Repair in Human Cancer-Prone Genetic Diseases		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.:	K. H. Kraemer	Research Scientist LMC NCI
Others:	S. Seetharam	Visiting Fellow LMC NCI
	H. Waters	Lab. Tech. (Biologist) LMC NCI
	M. Seidman	Guest Researcher LMC NCI
	D. Brash	Staff Fellow LHC NCI
	J. Scotto	Biometrician BB NCI
	G. Peck	Senior Investigator DB NCI
	M. Tucker	Clinical Investigator EEB NCI
COOPERATING UNITS (if any) Dept. of Pathology, New Jersey School of Medicine (W. C. Lambert); New York Blood Center (J. German); Dept. Dermatol., Hosp. of Univ. of PA (W.H. Clark); Dept. of Human Genetics, Tel Aviv University, Israel (H. Slor).		
LAB/BRANCH Laboratory of Molecular Carcinogenesis		
SECTION Nucleic Acids Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 3.5	PROFESSIONAL: 2.5	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input checked="" type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> Molecular, cellular and clinical abnormalities in patients with xeroderma pigmentosum (XP) and with the dysplastic nevus syndrome (DNS) of hereditary cutaneous melanoma are being studied. We have developed new assays utilizing plasmids as tools to measure DNA repair and mutagenesis at the molecular level. In DNA repair-deficient XP cells, we showed that one pyrimidine dimer blocks expression of a transfected gene. Enzymatic removal of pyrimidine dimers by photolyase demonstrated that nondimer photoproducts also block expression in XP cells. In normal human cells, UV pretreatment of a shuttle vector plasmid, pZ189, resulted in appearance of transition and transversion mutations. Survival of UV-treated pZ189 was reduced in the XP cells. There was a restricted mutagenic spectrum found in pZ189 replicated in XP cells; 93% of the base substitution mutations were GC to AT transitions ($p < 0.002$ vs normal). The major UV photoproduct, the thymine dimer, was only weakly mutagenic. We found that cultured lymphoblastoid cells from familial DNS patients were hypermutable to UV treatment. In a retrospective study of more than 800 XP patients, we have shown that they have a greater than 1000-fold increased risk of developing basal cell or squamous cell carcinoma or melanomas of the skin. The reported median age of first skin cancer was less than 10 years, a 50-year reduction in comparison to the United States general population. A prospective registry of xeroderma pigmentosum patients has been established. A clinical trial of skin cancer prevention in XP patients is in progress studying oral 13-cis retinoic acid as a possible chemopreventive agent. </p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

K. Kraemer	Research Scientist	LMC NCI
S. Seetharam	Visiting Fellow	LMC NCI
H. Waters	Lab. Tech. (Biologist)	LMC NCI
M. Seidman	Guest Researcher	LMC NCI
D. Brash	Staff Fellow	LHC NCI
J. Scotto	Biometrician	BB NCI
G. Peck	Senior Investigator	DB NCI
M. Tucker	Clinical Investigator	EEB NCI

Objectives:

Human cancer-prone genetic diseases are being studied with a view toward identifying groups of people with an increased susceptibility to environmental carcinogenesis. We are attempting to (1) understand the molecular basis of their cellular hypersensitivity, (2) correlate cellular hypersensitivity with clinical abnormalities, (3) determine if there is genetic diversity within such groups, (4) explore methods of cancer prevention in these patients, and (5) educate the medical community as to the importance of early recognition and diagnosis of these disorders.

Methods Employed:

Plasmids for measurement of DNA repair or of mutagenesis are treated in vitro with ultraviolet radiation, with acid and heat, and/or with damage-modifying enzymes. The extent and sites of damage are assessed by endonuclease-sensitive site assay or by polymerase stop assay with DNA sequencing. DNA-mediated gene transfer (transfection) is used to introduce the plasmids into cultured human cells. DNA repair is measured by transient expression of the encoded bacterial genes (CAT or XGPRT). Plasmid mutation frequencies are measured by isolation of replicated plasmids from the human cells and transformation of indicator strains of bacteria. Mutant plasmids are isolated from bacterial colonies and purified. The DNA sequence of mutated plasmids is determined using a primer-directed dideoxy sequencing technique with AMV reverse transcriptase on the double stranded plasmids.

DNA repair in cultured cells was measured by autoradiography, by scintillation spectroscopy or by alkaline elution. Mutagenesis of lymphoblastoid cells was measured by determination of the frequency of 6-thioguanine resistant cells in a microtiter well assay.

Patients with xeroderma pigmentosum (XP) or with DNS are examined with particular emphasis on cutaneous abnormalities. Cultures of skin fibroblasts or peripheral blood lymphocytes are established for laboratory analysis. The medical literature on XP is reviewed comprehensively, and information on individual patients is abstracted and entered into a computer for analysis. Physicians treating patients with XP have been contacted and are encouraged to fill out a Xeroderma Pigmentosum Registry questionnaire about their patients. New clinical

forms of XP are investigated in depth. XP patients with multiple cutaneous neoplasms are being treated with oral 13-cis retinoic acid in a 3-year study to attempt to reduce the rate of their new skin tumor formation.

Major Findings:

We found that transformed XP cells, but not primary fibroblasts, are suitable recipients of transfected genes in plasmids. XP cells were transfected with a plasmid containing two genes: chloramphenicol acetyl transferase (CAT) and xanthine phosphoribosyl transferase (XPRT). Eighty to 90% of the clones selected for ability to express XPRT also expressed CAT. Despite lower levels of expression, the XP cells integrated greater numbers of copies of the transfected genes than normal cells.

We developed a host cell reactivation assay using expression vector plasmids to measure DNA repair in XP cells. Transient expression of a UV-damaged plasmid depends on the competence of cellular repair enzymes. There were 100-fold differences in expression between normal and XP groups A cells. UV inactivation curves for three plasmids with different sizes and promoters containing the same gene were similar indicating that the target was the transfected gene. In XP cells, one pyrimidine dimer inactivated expression of the transfected gene. Selective removal of pyrimidine dimers by pretreatment with photoreactivating enzyme revealed that XP cells also cannot repair nondimer photoproducts. XP cells handle apurinic (AP) sites in plasmids in the same manner as do normal cells. Butyrate stimulates plasmid expression to a greater extent on a UV-damaged template than on an undamaged template in both normal and XP cells. This implies that butyrate does not specifically reverse the XP DNA repair defect.

The shuttle vector plasmid, pZ189, was used to measure replication and mutagenesis after UV treatment and transfection in normal and in XP cells. Plasmid survival was reduced in the XP cells reflecting their repair deficiency. The mutation frequency increased 100-fold with UV exposure. We demonstrated for the first time that there is a markedly reduced spectrum of mutations found with the XP cells in comparison to the spectrum in normal cells. There was a significant reduction in frequency of multiple base substitutions and in transversion mutations ($p < 0.002$). Ninety-three percent of the base substitutions were GC to AT ($p < 0.002$) with a large reduction in the number of sites of mutations in the XP cells. The major UV photoproduct, the thymine dimer, was only minimally mutagenic in normal or XP cells. The subset of the spectrum of mutations common to XP and normal may be related to ultraviolet induction of skin cancer.

Photoreactivation of the plasmid prior to transfection to selectively remove pyrimidine dimers resulted in increased plasmid survival, decreased mutation frequency, and altered mutational spectrum. This is the first demonstration that pyrimidine dimers (especially CC dimers) are mutagenic in mammalian cells.

Lymphoblastoid cell lines from patients with familial DNS were found to be hypermutable to UV treatment. Cell survival, rate of DNA synthesis, and rate of DNA repair as measured by formation of DNA strand breaks following UV treatment were normal. The finding of hypermutability of noncutaneous cells in DNS implies that DNS is a systemic disorder. This finding is of special importance

since DNS is only the second hypermutable human disease to be recognized. Studies of UV mutagenesis with the shuttle vector plasmid, pZ189, in DNS cells are in progress.

We compiled the most comprehensive review of the world literature to date on XP including both clinical and laboratory observations. Data on more than 800 patients were entered into the computer. In this retrospective study we documented a reduction of more than 50 years in the age of onset of skin neoplasms in XP in comparison to the United States population. There was a 1000-fold increase in all three major types of skin neoplasms: basal cell carcinomas, squamous cell carcinomas and melanoma. This study demonstrated the largest decrease in age of onset of cancer and increase in cancer frequency for any recessive human disorder. This implies that DNA repair plays a major role in protection against certain cancers. Neoplasms of the tip of the tongue were markedly increased probably due to UV exposure of the anterior tongue. An XP Registry has collected information on 100 patients. Seven XP patients with documented multiple skin cancers are being treated with oral 13-cis retinoic acid to prevent new skin cancers. This 3-year study is in progress.

Publications:

Andrews, A. and Kraemer, K. H.: Xeroderma pigmentosum. In Hornbliss, A. (Ed.): Ophthalmic and Orbital Plastic and Reconstructive Surgery. Baltimore, Williams and Wilkins (In Press).

Bredberg, A., Kraemer, K. H., and Seidman, M. M.: Restricted mutational spectrum in an UV-treated shuttle vector propagated in xeroderma pigmentosum cells. Proc. Natl. Acad. Sci. USA. (In Press)

Kraemer, K. H.: Cellular hypersensitivity and DNA repair. In Fitzpatrick, T. B., Eisen, A. Z., Wolff, K., Freedberg, I. M., and Austen, K. F. (Eds.): Dermatology in General Medicine. New York, McGraw-Hill (In Press).

Kraemer, K. H.: Heritable diseases with increased sensitivity to cellular injury. In Fitzpatrick, T. B., Eisen, A. Z., Wolff, K., Freedberg, I. M., and Austen, K. F. (Eds.): Dermatology in General Medicine. New York, McGraw Hill (In Press).

Kraemer, K. H., Protic-Sabljic, M., Bredberg, A., and Seidman, M. M.: Plasmid vectors for study of DNA repair and mutagenesis. In Wuepper, K. (ed.), Antenatal Diagnosis of Heritable Dermatoses. Basel, Karger (In Press).

Lambert, W. C., Andrews, A. D., German, J., and Kraemer, K. H.: Etiology and pathogenesis of xeroderma pigmentosum. In Dobson, R. L. and Thiers, B. H. (Eds.): The Etiology of Skin Diseases. New York, Churchill-Livingston Co. 1986, pp. 579-599.

Perera, M., Um, K. I., Greene, M. H., Waters, H. L., Bredberg, A., and Kraemer, K. H.: Hereditary dysplastic nevus syndrome: Lymphoid cell hypermutability in association with increased melanoma susceptibility. Cancer Res. 46: 1005-1009, 1986.

Protic-Sabljić, M., and Kraemer, K. H.: One pyrimidine dimer inactivates expression of a transfected gene in xeroderma pigmentosum cells. Proc. Natl. Acad. Sci. USA 82: 6622-6626, 1985.

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Protic-Sabljić, M., Tuteja, N., Munsen, P., Hauser, J., Kraemer, K. H., and Dixon, K.: UV-induced cyclobutane pyrimidine dimers are mutagenic in mammalian cells. Mol. Cell Biol. (In Press).

Protic-Sabljić, M., Whyte, D. B., Fagan, J., Howard, B. H., Gorman, C. M., Padmanabhan, R., and Kraemer, K. H.: Quantification of expression of linked cloned genes in an SV40 transformed xeroderma pigmentosum cell line. Mol. Cell Biol. 5: 1685-1693, 1985.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP04785-16 LMC																				
PERIOD COVERED October 1, 1985 through August 2, 1986																						
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) DNA Repair Studies on Human and Mouse Normal, Tumor, and Transformed Cells																						
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 40%;">R. S. Day, III</td> <td style="width: 30%;">Research Physical Scientist</td> <td style="width: 10%;">LMC</td> <td style="width: 10%;">NCI</td> </tr> <tr> <td>Others:</td> <td>M. Babich</td> <td>Staff Fellow</td> <td>LMC</td> <td>NCI</td> </tr> <tr> <td></td> <td>G. K. Townsend</td> <td>Biologist</td> <td>LMC</td> <td>NCI</td> </tr> <tr> <td></td> <td>T. Crook</td> <td>Visiting Fellow</td> <td>LMC</td> <td>NCI</td> </tr> </table>			PI:	R. S. Day, III	Research Physical Scientist	LMC	NCI	Others:	M. Babich	Staff Fellow	LMC	NCI		G. K. Townsend	Biologist	LMC	NCI		T. Crook	Visiting Fellow	LMC	NCI
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	G. K. Townsend	Biologist	LMC	NCI																		
	T. Crook	Visiting Fellow	LMC	NCI																		
COOPERATING UNITS (if any) Chemical Carcinogenesis Program, Litton Bionetics, Inc., Frederick, MD (D. Scudiero); Laboratory of Molecular Genetics, NICHD, NIH, Bethesda, MD (H. Okayama).																						
LAB/BRANCH Laboratory of Molecular Carcinogenesis																						
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TOTAL MAN-YEARS: 2.2	PROFESSIONAL: 2.2	OTHER: 0.0																				
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) Human subjects</td> <td><input checked="" type="checkbox"/> (b) Human tissues</td> <td><input type="checkbox"/> (c) Neither</td> </tr> <tr> <td><input type="checkbox"/> (a1) Minors</td> <td></td> <td></td> </tr> <tr> <td><input type="checkbox"/> (a2) Interviews</td> <td></td> <td></td> </tr> </table>			<input type="checkbox"/> (a) Human subjects	<input checked="" type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither	<input type="checkbox"/> (a1) Minors			<input type="checkbox"/> (a2) Interviews													
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SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> The study of human cells defective in repairing damaged DNA was extended, with the rationale that DNA repair-deficient cells are more susceptible to the adverse effects of carcinogens (cell killing, mutagenesis, sister chromatid exchange, and malignant transformation) than their repair-proficient counterparts. A group of 19 human tumor and eight SV40-transformed strains almost totally deficient in the repair of O-6-methylguanine (O-6-MeG, a modified DNA base made by certain methylating agents) was identified earlier in this project. Such strains are called Mer-. Transformation by SV40, Rous sarcoma virus, adenovirus, or Epstein-Barr virus produces Mer- strains. Mer+ but not Mer- strains contain about 60,000 copies of a 22,000 MW O-6-MeG-DNA methyl-transferase (O-6-DMT) that is responsible for repairing O-6-MeG by demethylation. Cell strains having intermediate amounts of O-6-DMT also have intermediate sensitivity to killing by 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) or by 1-(2-chloroethyl)-1-nitrosourea (CNU) and intermediate sensitivity to the induction of sister chromatid exchanges by MNNG. Human interferons alpha and beta inactivate Mer- tumor strains, while Mer+ human tumor strains are more resistant to such treatment, indicating an association between defective repair of O-6-MeG and sensitivity to interferons. We found O-6-MeG to be lethal to human cells under certain conditions. </p>																						

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

R. S. Day, III	Research Physical Scientist	LMC	NCI
M. Babich	Staff Fellow	LMC	NCI
G. K. Townsend	Biologist	LMC	NCI
T. Crook	Visiting Fellow	LMC	NCI

Objectives:

To learn more about DNA repair mechanisms in human and other mammalian cells and about their role in carcinogenesis. In particular, to determine the nature of the DNA repair defects both in human tumor cells and in cells from persons who are genetically predisposed to cancer. In addition, to use human cell strains with characterized defects to study the mechanisms of action of carcinogenesis or suspect carcinogens, chemotherapeutic agents, and other chemicals, in altering either DNA or the repair of damaged DNA.

Methods Employed:

1. Plaque assay: An adenovirus-host cell reactivation assay, developed previously in this project, was used to quantitate the deleterious effects of various chemical and physical treatments on the ability of the virus to initiate and sustain infection. The method involves establishing monolayer cell cultures which are infected with treated or nontreated adenovirus. The infected cells are then incubated 12-14 days with feeding by means of periodic overlaying with a nutrient agar. Nontreated virions or those treated ones which have been "reactivated" by cellular repair mechanisms form plaques of dead, lysed cells which are then counted.
2. Cellular extracts and partially purified fractions were assayed for O⁶-methylguanine-DNA methyltransferase repair activity using as a substrate either DNA methylated by [³H-methyl]-methylnitrosourea (MNU) or a synthetic double-stranded polymer (kindly supplied by Dr. S. Mitra, Oak Ridge National Laboratory) containing [³H-guanine]-O⁶-methylguanine. Reaction mixtures were incubated at 37°C, then heated in acid to remove purines, which were separated by high pressure liquid chromatography (HPLC) and quantified by liquid scintillation counting. A reduction of the O⁶-methylguanine to guanine ratio or a conversion of O⁶-methylguanine to guanine was interpreted to mean that repair of O⁶-methylguanine had occurred.
3. The survival of cells treated with DNA-damaging chemicals was assayed by growth of the cells into colonies of at least 50 cells. When the inhibitor of poly ADP ribose polymerase, 3AB, was used, it was prepared in complete medium and used to treat cells for one hour before application of the DNA-damaging chemical to the cells.
4. Plasmid production and purification together with transfer to E. coli and human cells, and assays for their presence followed published protocols.

5. The production and repair of methylated purines in DNA was followed using HPLC techniques, with either carbon-18 or strong cation exchange columns to separate both methylated bases and methylated deoxyribonucleosides.

Major Findings:

A major part of this year's research concerned the mechanisms involved in repair of DNA damage produced by alkylating agents (principally methylating agents), including chloroethylnitrosoureas (CNUs). Earlier in this project, we identified a group of 19 (of 93) human tumor cell strains that is unable to repair adenovirus damaged by MNNG. This repair-deficient phenotype we have termed Mer⁻. Human tumor cells having the Mer phenotype fail to repair O⁶-methylguanine (O⁶MeG), lack a 22,000 MW protein called O⁶MeG-DNA methyltransferase (O⁶DMT) and are extremely sensitive to MNNG in terms of both post-MNNG colony-forming ability and post-MNNG sister chromatid exchange (SCE) production. They fail to repair DNA:DNA cross-links produced by chloroethylating nitrosoureas and are easily killed by these agents.

Normal appearing fibroblasts of two patients whose tumors gave rise to Mer⁻ strains were determined to be Mer⁺. Moreover, Mer⁻ human tumor cells show more post-MNNG DNA repair synthesis but less post-MNNG semiconservative DNA synthesis than do human fibroblasts, and fail to restore control tertiary structure to their DNA after MNNG treatment.

We have also identified a group of five Mer⁺ strains that, while more resistant than Mer⁻ strains to MNNG in terms of post-treatment colony-forming ability, are about threefold (slope difference) more MNNG-sensitive than are 13 human fibroblast strains or other Mer⁺ strains. Rem⁺, for resistance to MNNG, is the phenotype we have assigned to human fibroblasts, so that the MNNG-sensitive Mer⁺ strains are Mer⁺ Rem⁻. Mer⁺ Rem⁻ cells behave as if they contain about one-third of the O⁶DMT complement present in Mer⁺ Rem⁺ cells.

Mer⁻ strains are produced from Mer⁺ strains by SV40 transformation, but these strains are Rem⁺. However, transformation by sarcoma virus gives rise to Mer⁻ Rem⁻ transformed strains. Adenovirus and Epstein-Barr virus transformation is also associated with Mer⁻ phenotype production.

Thus, in human tumor lines, Mer function (i.e., O⁶DMT) may be shut off by oncogene activity or is produced in them after infection by an adventitious virus, also present in the tumor biopsy. We have identified two groups of human cells having the Mer⁻ Rem⁺ phenotype, i.e., they fail to repair MNNG-damaged adenovirus (and fail to repair O⁶MeG), but are resistant in terms of post-MNNG colony-forming ability as are normal human fibroblasts. The groups include four Mer⁻ cell strains obtained by SV40 transformation and a number of MNNG-resistant revertants of the A1235 Mer⁻ human astrocytoma cell strain. However, strains of the Mer⁻ Rem⁺ phenotype are as sensitive as Mer⁻ Rem⁻ to inactivation by CNU or 3-hydroxyethyl-1-chloroethyl-1-nitrosourea (HECNU), believed to be lethal due to O⁶-chloroethylguanine production and (if not repaired) consequent DNA:DNA cross-link formation. Thus, the Mer⁻ phenotype correlates with lack of O⁶MeG repair activity, increased susceptibility to MNNG-produced SCEs, and sensitivity to killing by CNU or HECNU, but not with sensitivity to killing by MNNG.

Six strains of Mer⁻ human tumor cells are more sensitive to killing by human beta interferon (HuIFN-beta) than are 10 strains of Mer⁺ human tumor strains. Similar data was obtained with HuIFN-alpha. Unlike MNNG treatment, HuIFN treatment produced no O⁶MeG in DNA and produced no sister chromatid exchanges in Mer⁻ cells. Thus, HuIFNs may inactivate genes responsible for the unrestricted growth in many Mer⁻ strains but do not in Mer⁺ tumor strains. During tumorigenesis, activation of growth promoting oncogenes in Mer⁻ cells would be causatively linked with a shut off of Mer function. To test this hypothesis, we selected a mouse cell system in which a known oncogene is responsible for transformation and in which the oncogene is revertible by treatment with interferon. The transformed cells had deficient O⁶DMT, sensitivity to chloroethylnitrosourea (CNU), and an activated human c-Ha-ras 1 oncogene. We prepared three flat revertants using interferon: like the nontransformed NIH 3T3 cells, two had reduced transcription of the oncogene, elevated O⁶DMT, and sensitivity to CNU; the third, like the transformed RS 485 cells, had an activated oncogene and indicators of reduced repair capacity. The data are suggestive of a link between oncogene activation, repair of O⁶MeG, and the mode of interferon action.

We have shown that the O⁶DMT which repairs O⁶MeG in DNA also repairs the free base O⁶MeG at about a 40 million-fold lower rate. As a result, a two hour treatment of Mer⁺ cells with 2mM O⁶MeG (free base) reduces O⁶DMT activity because the protein acts stoichiometrically and is inactivated by its substrate. Mer⁺ cells treated in this way are more sensitive to killing by CNU than are non-treated cells. The CNU sensitivity of Mer⁻ cells is little affected by the pretreatment, showing that repair cannot be blocked in a case in which little repair is possible, supporting our interpretation.

The sensitivity to killing by MNNG of cells pretreated with free O⁶MeG is also enhanced but only if the MNNG-treated cells receive a free O⁶MeG post-treatment as well. We have shown that the repair of O⁶MeG in cellular DNA occurs extensively in cells that received the O⁶MeG pretreatment, and believe that continued depletion of rapidly synthesized O⁶DMT is required to potentiate lethality by MNNG. (Continued depletion is not required to potentiate killing due to CNU because, once formed, DNA cross-links produced by CNU are not reversible by O⁶DMT). Thus, it appears that in addition to being a mutagenic and SCE-producing lesion, O⁶MeG can be a lesion lethal to human cells.

Publications.

Day, R. S., III, Babich, M. A., Yagi, T., Heilman, C. A., Yarosh, D. B., and Scudiero, D. B.: The relationship of repair of O⁶-methylguanine to interferon action and oncogene activation. Proceedings of Symposium on the Repair of Damage due to N-Nitrosocompounds. Tromso, Norway, June 1985 (In Press).

Yarosh, D. B., Fornace, A., and Day, R. S., III: Repair of O⁴-methylthymine and methyl phosphotriesters in DNA by human O⁶-alkylguanine alkyltransferase. Carcinogenesis 6: 949-953, 1985.

Yarosh, D. B., Hurst-Calderone, S., Babich, M. A., and Day, R. S., III: Inactivation of O⁶-methylguanine-DNA methyltransferase and sensitization of human tumor cells to killing by chloroethylnitrosourea by O⁶-methylguanine as a free base. Cancer Res. 46: 1663-1668, 1986.

Yarosh, D. B., Yagi, T., Day, R. S. III, and Scudiero, D. A.: Human tumor strains unable to repair O⁶-methylguanine and hypersensitivity to killing by human alpha- and beta-interferons. Carcinogenesis 6: 883-886, 1985.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z010P05086-08 LMC
PERIOD COVERED October 1, 1986 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Preparation and Characterization of Monoclonal Antibodies to Human Cytochrome P-450		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.:	S. S. Park	Expert LMC NCI
Others:	H. V. Gelboin	Chief LMC NCI
	I. Y. Ko	Guest Researcher LMC NCI
	G. M. Sundaresan	Chemist LMC NCI
	T. X. Hamilton	Biological Aid LMC NCI
COOPERATING UNITS (if any) Univ. of Oulu, Finland (O. Pelkonen); Vanderbilt Univ., School of Medicine, Nashville, TN (F. P. Guengerich); Hebrew Univ., Israel (H. Kapultunik).		
LAB/BRANCH Laboratory of Molecular Carcinogenesis		
SECTION Metabolic Control Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20982		
TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 0.5	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Metabolism and sensitivity to drugs and chemical carcinogens differ among tissues, organs, individuals and species. The type and quantity of specific forms of cytochrome P-450 determine the extent of activation and/or detoxification of particular substrates. Monoclonal antibodies (MAbs) are specific probes for cytochrome P-450 isozymes. The MAbs to rat cytochrome P-450 which cross-react with human cytochromes P-450 are useful tools for identification of particular isozymes. MAbs to pregnenolone 16-α-carbonitrile- and ethanol-inducible rat cytochromes P-450 detect cytochrome P-450 in human liver. MAbs to human cytochromes P-450 from placenta, mitochondria and liver were prepared. These MAbs may be useful in phenotyping human cytochrome P-450 in different tissues and organs of individuals exposed to different environments. The roles of different forms of cytochrome P-450 in the metabolism of chemical carcinogens can then be evaluated.</p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. S. Park	Expert	LMC	NCI
H. V. Gelboin	Chief	LMC	NCI
I. Y. Ko	Guest Researcher	LMC	NCI
G. M. Sundaresan	Chemist	LMC	NCI
T. X. Hamilton	Biological Aid	LMC	NCI

Objectives:

To construct a library of monoclonal antibodies (MAbs) to human cytochromes P-450, as well as MAbs to animal cytochromes P-450 which cross-react with human isozymes. The MAbs will be used to identify and examine the role of cytochromes P-450 in chemical carcinogenesis and will be useful in defining individual differences in drugs and carcinogen metabolism.

Methods Employed:

MAbs prepared to animal cytochromes P-450 are examined for their cross-reactivities with purified human cytochrome P-450 or human liver microsomal preparations. MAbs to cytochrome P-450 of human tissues and organs are prepared by hybridization of myeloma cells with spleen cells derived from mice which were immunized with a specific human cytochrome P-450. The identification and characterization of cytochromes P-450 with MAbs are carried out by RIA and Western blotting using different tissues and organs.

Major Findings:

MAbs to pregnenolone 16- α -carbonitrile- and ethanol-induced rat cytochrome P-450 cross-react with cytochromes P-450 in human liver microsomal preparations. MAbs to human liver, placenta, and mitochondrial cytochromes P-450 bind to their respective cytochromes P-450 and belong to a single mouse immunoglobulin type. The specificity of new MAbs in immunoprecipitation and cross-reactivity and their effect on catalytic activities are under investigation by RIA, Western blotting and activity in microsomal and reconstituted enzyme systems.

Publications:

Hietanen, E., Bartsch, H., Ahotupa, M., Park, S. S., and Gelboin, H.: Tissue specificity of extrahepatic monooxygenases in the metabolism of xenobiotics. In Siest, G. (Ed): Drug Metabolism: Molecular Approaches and Pharmacological Implications. New York, Pergamon Press, 1985, 141-146.

Pelkonen, O., Pasanen, M., Kuha, H., Gachalyi, B., Kairaluoma, M., Sotaniemi, E. A., Park, S. S., Friedman, F. K. and Gelboin, H. V.: The effect of cigarette smoking on 7-ethoxyresorufin O-deethylase and other monooxygenase activities in human liver: analyses with monoclonal antibodies. Br. J. Clin. Pharmacol. (In Press).

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP5109-07 LMC										
PERIOD COVERED October 1, 1985 to September 30, 1986												
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Chromatin Structure and Carcinogenesis												
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 30%;">Michael Bustin</td> <td style="width: 30%;">Acting Section Chief</td> <td style="width: 10%;">LMC</td> <td style="width: 10%;">NCI</td> </tr> <tr> <td>Others:</td> <td>Thyagarajan Srikantha</td> <td>Visiting Fellow</td> <td>LMC</td> <td>NCI</td> </tr> </table>			PI:	Michael Bustin	Acting Section Chief	LMC	NCI	Others:	Thyagarajan Srikantha	Visiting Fellow	LMC	NCI
PI:	Michael Bustin	Acting Section Chief	LMC	NCI								
Others:	Thyagarajan Srikantha	Visiting Fellow	LMC	NCI								
COOPERATING UNITS (if any) Department of Cell Biology, New York University School of Medicine (Dr. M. Adesnik)												
LAB/BRANCH Laboratory of Molecular Carcinogenesis												
SECTION Protein Section												
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892												
TOTAL MAN-YEARS: 1	PROFESSIONAL: 1	OTHER: 0										
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews												
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> The chromatin structure of genes coding for P-450 enzymes is being investigated. Changes in the chromatin structure upon gene activation is examined by comparing the chromatin structure of these genes in nuclei purified from either normal or carcinogen-treated rats. Micrococcal nuclease digestion revealed that, in the liver, genes coding for the 3-methylcholanthrene-inducible P-450c, P-450d, and P-450m enzymes are not organized in the typical 200 base pair nucleosomal conformation. DNase 1 hypersensitive sites were mapped in each of these genes. These sites were not observed in nuclei isolated from the thymus. These results suggest that in the liver the chromatin structure is altered to allow tissue-specific gene expression. Gene induction by carcinogen treatment is associated with a rearrangement of the nuclear location of the P-450c and P-450d genes and with a change in one of the DNase 1 hypersensitive sites present in the P-450c gene. The results suggest that gene induction is associated with detectable changes in the chromatin structure of these genes. </p>												

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged in this Project:

Michael Bustin	Acting Section Chief	LMC, NCI
Thyagarajan Srikantha	Visiting Fellow	LMC, NCI

Objectives:

To study the molecular mechanisms involved in the activation of carcinogen metabolizing enzymes by examining genomic changes associated with exposure of organisms to chemical carcinogens.

Methods Employed:

Exposure of organisms to a variety of xenobiotics and drugs induces the synthesis of enzymes belonging to the P-450 family of mixed function oxidases. Induction of gene expression often is correlated with detectable changes in the chromatin structure of the inducible genes. Elucidation of the chromatin structure of the enzymes involved in the metabolism of drugs and carcinogens may help in understanding the molecular mechanisms involved in carcinogenesis. We have concentrated our efforts on studying the chromatin structure of two genes inducible by 3-methylcholanthrene and genes inducible by phenobarbital. Genomic clones were subcloned in pBR322 to give probes which can be radioactively labelled by nick translation or primer extension and used in Southern blots to analyze the structure of the gene. Nuclei were isolated from the liver of control and carcinogen-treated rats, the DNA isolated and restricted, and the restriction fragments separated on agarose gels and transferred to a solid support.

Digestion of nuclei with micrococcal nuclease and examination of the DNA isolated from such nuclei allows determination of the nucleosomal structure of the gene. Examination of the Southern hybridization pattern with DNA isolated from DNase I digested nuclei allows mapping of the DNase I sensitive sites in the gene. The structure of both the inducible and noninducible P-450 genes from both inducible and noninducible tissues can be examined. S1 sensitive sites are mapped in plasmids containing various subclones of the gene. The plasmids are cut with S1 under various conditions, the digested plasmid re-restricted and the S1 site mapped by Southern analysis with known probes.

Major Findings:

In this study, the chromatin structure of three types of genes was investigated. Gene P-450m codes for a constitutive P-450 enzyme whose synthesis is induced fourfold upon treatment of rats with 3-MC. Gene P-450c codes for an enzyme whose synthesis is induced over 100-fold by the same treatment. Genes P-450b and P-450e code for phenobarbital inducible enzyme.

P-450m is a minor P-450 species which is constitutively expressed. Treatment of rats with the chemical carcinogen 3-methylcholanthrene results in a four-fold increase in the transcription of this gene. The coding regions of the

P-450m gene are not organized in a typical 200 base pair nucleosomal conformation and are more sensitive to digestion with DNase 1 than bulk chromatin. In liver, where this gene is expressed, there are four DNase 1 hypersensitive sites in the 5' region of the gene. Gene induction does not change the location of these sites. Rat thymus does not contain these sites, suggesting that in the liver the chromatin structure of this gene is altered to allow tissue-specific expression. Treatment of rats with 3-methylcholanthrene induces, in the liver, two major isoenzymes designated as P-450c and P-450d. In the liver these genes are not present in the typical nucleosomal organization. Using indirect end-label hybridization, three DNase 1 hypersensitive sites were mapped in this gene. Gene induction is associated with a rearrangement of the nuclear organization of these genes and brings about a change in the location of one of the DNase 1 hypersensitive sites. Thus, gene induction is associated with some type of remodeling in the chromatin structure. The chromatin structure of these genes is different in the thymus suggesting that tissue-specific expression is associated with a particular chromatin conformation.

Using antibodies specific for DNA modified by benzo-(α)-pyrene diol epoxide, we found that the binding of the carcinogen to the DNA in polytene chromosomes is site specific.

Publications:

Einck, L., Fagan, J. and Bustin, M.: The chromatin structure of a 3-methylcholanthrene induced P-450 gene. Biochemistry 24: 5269-5275, 1985.

Kurth, P. and Bustin, M.: Site specific carcinogen binding to DNA in polytene chromosomes. Proc. Natl. Acad. Sci. USA 82: 7076-7081, 1985.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05125-06 LMC	
PERIOD COVERED October 1, 1985 to September 30, 1986			
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Preparation of Monoclonal Antibodies and Epitope Typing of Cytochrome P-450			
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)			
PI:	S. S. Park	Expert	LMC NCI
Others:	H. V. Gelboin	Chief	LMC NCI
	I. Y. Ko	Guest Researcher	LMC NCI
	G. M. Sundaresan	Chemist	LMC NCI
	T. X. Hamilton	Biological Aid	LMC NCI
COOPERATING UNITS (if any) UMDJ-New Jersey Medical School, Newark, NJ (C. S. Yang); Dana-Farber Cancer Institute, Boston, MA (D. J. Waxman); Woods Hole Oceanographic Inst., Woods Hole, MA (J. Stegeman); Vanderbilt Univ., School of Medicine, Nashville, TN (F. P. Guengerich).			
LAB/BRANCH Laboratory of Molecular Carcinogenesis			
SECTION Metabolic Control Section			
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892			
TOTAL MAN-YEARS:		PROFESSIONAL:	OTHER:
2.0		0.5	1.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews			
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> Cytochromes P-450 are the key components of the mixed-function oxidase system which metabolizes most chemical carcinogens, including benzo(a)pyrene, aflatoxin, nitrosamine and protein-pyrollysates. Specific forms of cytochromes P-450 are responsible for the activation and disposition of chemical carcinogens. Therefore, identification and quantitation of cytochrome P-450 isozymes are essential to understand the role of cytochrome P-450 in the primary stages of chemical carcinogenesis and in individual differences in sensitivity to chemical carcinogens. Our approach is to prepare and use monoclonal antibodies (MAbs) as specific probes for individual and classes of cytochrome P-450. Panels of MAbs have been prepared to cytochromes P-450 of rats treated with phenobarbital, 3-methylcholanthrene, pregnenolone 16-α-carbonitrile and ethanol, and an environmentally induced cytochrome P-450 in fish (scup). These MAbs are characterized for their epitope specificity toward different cytochrome P-450 isozymes. A MAb to the fish cytochrome P-450 cross-reacts with 3-methylcholanthrene-inducible rat cytochrome P-450 and may be useful for monitoring polluted marine environments. MAbs to an ethanol-inducible and nitrosamine metabolizing cytochrome P-450 (P-450et) have been utilized for identification of P-450et in liver microsomal preparations of rats which were untreated or treated with different chemical agents. </p>			

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. S. Park	Expert	LMC	NCI
H. V. Gelboin	Chief	LMC	NCI
I. Y. Ko	Guest Researcher	LMC	NCI
G. M. Sundaresan	Chemist	LMC	NCI
T. X. Hamilton	Biological Aid	LMC	NCI

Objectives:

Chemical carcinogens which are encountered in the environment [e.g. benzo(a)-pyrene] and foods (e.g., aflatoxin, nitrosamine and protein pyrolysates) are activated to ultimate carcinogens by mixed function oxidase systems. Cytochrome P-450, a key component of the system, is synthesized constitutively or by induction with specific inducers. We have applied hybridoma technology to prepare and characterize monoclonal antibodies (MAbs) to specific cytochromes P-450. The MAbs are useful for phenotyping cytochromes P-450 of animals and humans, and for measuring individual differences in sensitivity toward drugs and chemical carcinogens.

Methods Employed:

Balb/c female mice were immunized with purified cytochrome P-450 of rats treated with ethanol (P-450et). The primed spleen cells were isolated and fused with myeloma cells, using polyethylene glycol. The hybrid cells were grown in a selective medium (HAT) and screened by RIA to identify hybridomas producing MAbs to P-450et. The specificity of MAbs was determined by RIA, double immunodiffusion analyses and Western blotting. The effect of MAbs on P-450et catalytic activity was also measured in microsomal and reconstituted reaction systems.

Major Findings:

1) Thirty one independent hybridomas producing MAbs to P-450et were obtained. The MAbs belonged to several groups which are distinctive in immunoglobulin subclass types, binding to P-450et, immunoprecipitation, Western blotting and inhibitory effect on P-450et catalytic activity. 2) When 10 representative MAbs were tested for their specificity toward 18 cytochrome P-450 isozymes, only five MAbs were specific for P-450et. One of these MAbs was utilized to identify P-450et in liver microsomal preparations by RIA and Western blotting. 3) Only one of 31 MAbs had strong inhibitory effect on aniline p-hydroxylase and nitrosodimethyl-amine-demethylase activities in both microsomal and reconstituted systems.

Publications:

Hietanen, E., Malaveille, C., Friedman, F. K., Park, S. S., Bereziat, J., Brun, G., Bartsch, H., and Gelboin, H. V.: Monoclonal antibody-directed analysis of cytochrome P-450-dependent monooxygenases and mutagen activation in livers of DBA/2 and C57BL/6 mice. Cancer Res. 46: 524-531, 1986.

Park, S. S., Ko, I., Patten, C., Yang, C. S., and Gelboin, H.: Monoclonal antibodies to ethanol induced cytochrome P-450 that inhibit aniline and nitrosamine metabolism. Biochem. Pharmacol. (In Press).

Park, S. S., Miller, H., Koltz, A. V., Kloepper-Sam, P. J., Stegeman, J. J., and Gelboin, H. V.: Monoclonal antibodies to liver microsomal cytochrome P-450 of the marine fish Stenotomus chrysops (scup): Cross reactivity with 3-methylcholanthrene induced rat cytochrome P-450. Arch. Biochem. Biophys. (In Press).

Park, S. S., Waxman, D. J., Miller, H., Robinson, R., Attisano, C., Guengerich, F. P., and Gelboin, H. V.: Preparation and characterization of monoclonal antibodies to pregnenolone 16- α -carbonitrile. Biochem. Pharmacol. (In Press).

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05208-06 LMC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Phenotyping Of Human Cytochrome P-450		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Fred K. Friedman	Senior Staff Fellow LMC NCI
Others:	Haruko Miller	Bio. Lab. Tech. LMC NCI
	Sang S. Park	Expert LMC NCI
	Harry V. Gelboin	Chief LMC NCI
COOPERATING UNITS (if any) Hebrew University, Jerusalem, Israel (H. Kapitulnik) University of Oulu, Finland (O. Pelkonen)		
LAB/BRANCH Laboratory of Molecular Carcinogenesis		
SECTION Metabolic Control Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	.2	1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) The individual forms of cytochrome P-450 display unique substrate specificity and reactivity profiles toward a variety of drugs and carcinogens. Differences in cytochrome P-450 phenotype may relate to individual differences in sensitivity to certain drugs and susceptibility to carcinogenesis. Monoclonal antibodies (MAbs) to cytochromes P-450 have been used as specific probes for epitope-specific cytochromes P-450 in human liver samples obtained from different individuals. Western blot analysis with antisera or a MAb to rat ethanol-induced P-450 detected a P-450 in human liver homogenates and microsomes. The level of the immunodetectable P-450 varied among livers from different individuals. A human liver P-450 was immunopurified using the MAb and was structurally characterized. In addition, we are developing a radioimmunoassay for human cytochrome P-450 as a rapid, efficient method for screening large numbers of samples from human tissues.		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Fred K. Friedman	Senior Staff Fellow	LMC	NCI
Haruko Miller	Bio. Lab. Tech.	LMC	NCI
Sang S. Park	Expert	LMC	NCI
Harry V. Gelboin	Chief	LMC	NCI

Objectives:

The profile of cytochromes P-450 in a tissue may influence metabolism that results in either activation or detoxification of potential carcinogens. Immunochemical methods are utilized to evaluate the P-450 content of human tissues in order to relate P-450 phenotype to individual differences in sensitivity to drugs and carcinogens.

Methods Employed:

MAbs were prepared to several rat liver cytochromes P-450. Human liver microsomes or homogenates were applied to SDS-gels and subjected to Western blot analysis. MAbs or mouse antiserum to rat ethanol-induced P-450 (P-450et) was used for immunostaining. Intensity of stained protein was determined by densitometry. Competitive radioimmunoassay (RIA) was carried out using radio-labeled MAbs and coating microtiter wells with liver microsomes. Human liver P-450 was immunopurified from solubilized microsomes by immunopurification with MAb 1-98-1 to P-450et.

Major Findings:

Human liver biopsy homogenates, or microsomes from whole livers, were analyzed by Western blots using MAbs or antiserum to rat P-450et. The antiserum immunoreacted with a protein in the P-450 region in homogenates. MAb 1-98-1 detected immunoreactive protein in human liver microsomes. Differences in band intensity were observed among the individual samples.

A competitive RIA was developed using either rat or human liver microsomes to coat microtiter cells. Individual differences in competition were observed using MAbs to phenobarbital-induced rat P-450. These differences were more pronounced when human microsomes were used as the well-coating agent. A sensitive RIA for human P-450 using cross-reactive MAbs is being developed.

Immunopurification of P-450 from liver microsomes was carried out with a series of MAbs. Use of MAb 1-98-1 successfully immunopurified an electrophoretically homogeneous P-450 which was structurally characterized.

Publications:

None

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05318-04 LMC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Immunopurification and Characterization of Cytochrome P-450		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Fred K. Friedman	Senior Staff Fellow LMC NCI
Others:	Richard C. Robinson	Biologist LMC NCI
	Sang S. Park	Expert LMC NCI
	Harry V. Gelboin	Chief LMC NCI
COOPERATING UNITS (if any) National Institute of Aging, NIH (J. Rifkind).		
LAB/BRANCH Laboratory of Molecular Carcinogenesis		
SECTION Metabolic Control Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.7	0.7	1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> The cytochromes P-450 metabolize a wide array of compounds, including xenobiotics such as drugs and carcinogens, and endogenous compounds such as steroids. The focus of this project is the identification, characterization, and elucidation of structure-function relationships of the multiple forms of this enzyme. Monoclonal antibodies (MAbs) to rat P-450s are an important tool in these studies. 3-methylcholanthrene (MC)-inducible P-450s have been immunopurified from the livers of rats, C57Bl/6 and DBA/2 mice, guinea pigs and hamsters. Their primary structures were compared by amino acid sequence analysis and peptide mapping, and revealed varying degrees of homology. Several previously unidentified amino terminal residues of an immunopurified mouse P-450 were identified, and the sequence of a guinea pig liver P-450 was determined for the first time. A rat lung P-450 was also immunopurified and was indistinguishable from a liver P-450 by several criteria. Using a MAb to ethanol-inducible rat liver P-450, a P-450 has been purified from human liver and was structurally characterized. Since immunopurification typically denatured the P-450s, an alternative approach involving antigen-exchange was developed in which inactive denatured P-450 displaces active P-450 from the immunoadsorbent. Such epitope-specific exchange may be generally applicable to preparation of proteins with retention of activity. The epitopic structure of the P-450 surface was mapped by evaluating the binding of MAbs to various P-450 peptide fragments and by computer-aided alignment of homologous P-450s which are epitopically related. P-450 dependent testosterone metabolism in 3- and 24-month old rats were examined, and hydroxylation patterns as well as content of liver constitutive P-450s varied with age. </p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Fred K. Friedman	Senior Staff Fellow	LMC	NCI
Richard Robinson	Biologist	LMC	NCI
Sang S. Park	Expert	LMC	NCI
Harry V. Gelboin	Chief	LMC	NCI

Objectives:

To identify, purify, and characterize the multiple forms of cytochrome P-450 in animal and human tissues. Monoclonal antibodies (MAbs) are utilized as highly specific reagents for distinguishing closely related forms of P-450 on the basis of epitope content.

Methods Employed:

MAbs were prepared to several liver P-450s from rats treated with inducing agents such as 3-methylcholanthrene (MC) and phenobarbital (PB) or acetone. The MAbs were covalently linked to Sepharose to yield an immunoadsorbent. When microsomes from various tissues and species were applied, the resin extracted MAb-specific P-450s, which were subsequently eluted at pH 3.0 and characterized. Analytical methods employed include gel electrophoresis, Western blots, spectral analyses, and measurements of aryl hydrocarbon hydroxylase (AHH) and 7-ethoxycoumarin deethylase (ECD) activities.

Major Findings:

Using immunoadsorbents based on the MAbs 1-7-1 and 1-31-2, both to MC-induced P-450, cytochrome P-450 was purified from MC-induced livers of rats, C57Bl/6 and DBA/2 mice, hamsters, and guinea pigs. Acid conditions (pH 3) were necessary to elute P-450 from the immunoadsorbents.

The immunopurified P-450s were analyzed for structural homology by peptide mapping studies with SDS-gels and HPLC, and by N-terminal amino acid analysis. The peptide patterns and sequences revealed varying degrees of structural homology among these P-450s. The sequences of five rat and mouse isozymes were consistent with previously reported sequences, with the exception that several N-terminal residues of a mouse P-450 were not previously identified. The guinea pig liver P-450 sequence was determined for the first time and exhibited the least homology among the isolated P-450s.

Extrahepatic P-450s have not been as extensively studied as the liver P-450s owing to their lower levels and difficulty in purification by conventional methods. A P-450 was MAb-immunopurified from rat lung and was indistinguishable from the liver form on the basis of apparent molecular weight, MC-inducibility, peptide maps on an SDS-gel, and NH₂-terminal sequence.

Using a MAb to rat ethanol-inducible P-450, P-450s were isolated from both rat and human liver microsomes and were structurally characterized.

Our standard immunopurification procedure denatures the cytochrome P-450 during the desorption step and thus, does not yield catalytically active P-450. We have developed an antigen-exchange technique which entails displacement of immunoabsorbent-bound active P-450 by immunopurified, denatured P-450. This approach yielded P-450 which retained AHH and ECD activities.

The age dependence of P-450 phenotype was evaluated by comparing testosterone metabolism and P-450 levels in liver microsomes from 3- and 24-month old male rats. The pattern of ring hydroxylations as well as the level of several constitutive P-450s on Western blots were significantly different. Electron spin resonance spectra also revealed differences in the P-450 contents.

The epitopic structure of rat P-450s were investigated with MAbs to P-450s. Peptide fragments of P-450s were evaluated for reactivity with MAbs, and reactive fragments sequenced. This information, in conjunction with computer-aided alignment of homologous P-450s with a common epitope, was used to localize the region of a specific epitope on the P-450 surface.

Organization of P-450s in the microsomal membrane was studied by chemical cross-linking of associated membrane proteins and subsequent immunopurification with MAbs to P-450. The results suggested that the MAb-specific P-450 was not monomeric in the membrane, but was associated with other proteins.

Publications:

Cheng, K. C., Park, S. S., Krutzsch, H. C., Grantham, P. H., Gelboin, H. V. and Friedman, F. K.: Amino-terminal sequence and structure of monoclonal antibody immunopurified cytochromes P-450. Biochemistry 25: 2397-2402, 1986.

Friedman, F. K., Park, S. S., Song, B. J., Cheng, K. C. and Gelboin, H. V.: Monoclonal antibody-directed analysis of cytochrome P-450. In D. J. Jollow, J.O. Nelson and R. Snyder (Eds.): Biological Intermediates III: Mechanisms of Action in Animal Models and Human Disease. New York, Plenum (In Press).

Friedman, F. K., Robinson, R. R., Song, B. J., Park, S. S. and Gelboin, H. V.: Preparation of catalytically active cytochromes P-450 by antigen exchange on monoclonal antibody based immunoabsorbents. Biochemistry 24: 7044-7048, 1985.

Robinson, R. C., Cheng, K. C., Park, S. S., Gelboin, H. V. and Friedman, F. K.: Structural comparison of immunopurified pulmonary and hepatic cytochrome P-450 from 3-methylcholanthrene treated rats. Biochem. Pharmacol. (In Press).

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05436-02 LMC
PERIOD COVERED October 31, 1985 through September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <u>The Role of Cytochrome P-450 Enzymes in Carcinogenesis</u>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.:	N. Battula	Expert LMC NCI
Others:	J. Sagara	Visiting Fellow LMC NCI
	F. Gonzalez	Senior Staff Fellow LMC NCI
	H. V. Gelboin	Chief LMC NCI
COOPERATING UNITS (if any) NONE		
LAB/BRANCH Laboratory of Molecular Carcinogenesis		
SECTION Metabolic Control		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.) <p> Microsomal cytochrome P-450s are a family of enzymes, some of which metabolize foreign compounds and chemical carcinogens. Some of these enzymes are inducible by carcinogenic compounds. For example, 3-methylcholanthrene induces cytochromes P1-450 and P3-450 in the mouse. These induced enzymes metabolize benzo(a)pyrene, aryl amines and other carcinogens. Some of the active metabolites formed bind to DNA and thus are presumed to initiate mutagenesis and carcinogenesis. We propose to express the cytochrome P1-450 and P3-450 enzymes in homologous and heterologous cells and determine the contribution of these enzymes to mutation and cell transformation. </p> <p> For this purpose, we have constructed infectious recombinant vaccinia viruses containing the full length cDNAs of P1-450 and P3-450. Human and mouse cells infected with the recombinant viruses showed high level expression of the authentic proteins as detected by immunoblotting. The expressed proteins are enzymatically active and exhibit distinguishable substrate specificities. We have also constructed recombinant retroviruses containing the cytochromes P1-450 and P3-450. Cells infected with these recombinants express cytochrome P3-450. Preliminary experiments indicate that the protein is enzymatically active. This system is now amiable to mutation and transformation analysis. </p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

N. Battula	Expert	LMC	NCI
J. Sagara	Visiting Fellow	LMC	NCI
F. Gonzalez	Senior Staff Fellow	LMC	NCI
H. V. Gelboin	Chief	LMC	NCI

Objectives:

The overall goal of this project is to determine the contribution of the inducible cytochrome P-450 enzymes to chemical carcinogenesis. Specific aims are (a) to express the full length cDNA of inducible cytochrome P-450s in recombinant vaccinia viruses and recombinant retroviruses, (b) to test if the expressed proteins are enzymatically active and transported to the appropriate subcellular target site, (c) to test the extent of binding of the challenging carcinogen to cellular macromolecules and (d) to test for mutation rates and cell transformation after exposure to carcinogens of cells expressing the P-450 enzymes.

Methods Employed:

This research requires the construction of recombinant viruses. For these constructions, genetic engineering methods were used extensively. The infectious recombinant vaccinia viruses were generated within cells by homologous recombination and the infectious recombinant retroviruses were generated by complementation of missing viral functions provided by host cells. This part of the work involved use of cell culture procedures, DNA transfections, genetic selection and virological procedures. Detection of expressed products involved electrophoretic separation of proteins and their identification by immunoblotting using monoclonal and polyclonal antibodies. Localization of the expressed proteins involved the use of subcellular fractionations. Enzymatic activity of the proteins was determined by assaying for aryl hydrocarbon hydroxylase activity and acetanilide hydroxylase activity. The metabolites of these enzyme products were detected using highly sensitive fluorescence spectrophotometry and high pressure liquid chromatography.

Major Findings:

Infectious recombinant vaccinia viruses containing the full length cDNAs of 3-methylcholanthrene-induced mouse cytochrome P1-450 and P3-450 were constructed and characterized. Mouse cells and human cells infected with the vaccinia recombinants produced a large amount of proteins that comigrated with authentic P1-450 and P3-450, indicating high level expression of these proteins. The expressed P1-450 and P3-450 were enzymatically active. The specific aryl hydrocarbon hydroxylase activity of P1-450 was about 15-fold higher than that of the P3-450, a distinguishing feature of these enzymes. Infectious recombinant retroviruses of limited host-range (ecotropic) and wide host-range (amphotropic), containing the full length cDNAs of P1-450

and P3-450, were constructed and characterized. Immunoblotting analysis showed that these recombinants expressed authentic size P3-450. However, P1-450 was not detected. Preliminary experiments show that the cytochrome P3-450 is enzymatically active.

Publications:

None

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05474-01 LMC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Purification and Characterization of Rat and Rabbit Cytochrome P-450		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.:	Harry V. Gelboin	Chief LMC NCI
Others:	Sang S. Park	Expert LMC NCI
	Toshifumi Aoyama	Visiting Fellow LMC NCI
COOPERATING UNITS (if any) Institute for Protein Research, Osaka University, Osaka, Japan (R. Sato); Toneyama Inst. for Tuberculosis Res., Univ. Japan, Osaka, Japan (M. Kusunose)		
LAB/BRANCH Laboratory of Molecular Carcinogenesis		
SECTION Metabolic Control Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> The cytochromes P-450 metabolize exogenous materials such as carcinogens and drugs as well as endogenous ones such as cholesterol, fatty acids and prostaglandins. The focus of this project is the purification and characterization of these isozymes. Many forms of P-450 were purified from rat liver and kidney, and rabbit liver microsomes. These purified P-450s were characterized catalytically, structurally and spectrally, and classified on the basis of immunological cross-reactivity. Several forms of P-450 were used to study the conformation of the protein surface, using monoclonal antibodies (MAbs) to specific cytochromes P-450. MAbs were also used for immunopurification of P-450s with catalytic activity, for making a P-450-bioreactor and for evaluating epitope topography of the microsomal membrane. </p>		

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Harry V. Gelboin	Chief	LMC	NCI
Sang S. Park	Expert	LMC	NCI
Toshifumi Aoyama	Visiting Fellow	LMC	NCI

Objectives:

To purify, characterize and classify the multiple forms of cytochrome P-450 in rat and rabbit tissues; to elucidate structure-function relationships of these isozymes; to clarify tissue and animal specificity of P-450; to purify P-450 with catalytic activity, evaluate epitope topography and make a P-450-bioreactor.

Methods Employed:

Cytochrome P-450 were purified from liver microsomes of untreated, phenobarbital (PB)-treated and β -naphthoflavone (NF)-treated rats, liver microsomes of untreated, PB-, NF-, imidazole- and isosafrole-treated rabbits, and kidney microsomes of untreated and 3-methylcholanthrene (MC)-treated rats and rabbits by column chromatography. MABs were prepared to several rat P-450s. Catalytic activities were measured by using various exogenous chemicals, fatty acids, vitamin D₃ and prostaglandins as substrates. Cross-reactivity was measured by Ouchterlony double diffusion, radial immunodiffusion, radioimmunoassay (RIA) and ELISA. A noninhibitory MAB was covalently linked to agarose to yield an immunoadsorbent which was used both as a P-450-bioreactor and for immunopurification of P-450.

Major Findings:

Forty-four forms of P-450 have been purified and characterized in an ongoing project at Osaka University for the past three years. At least 26 forms displayed different properties from the others on the basis of their structural, spectral and catalytic properties.

Catalytic activities towards fatty acids, vitamin D₃ and prostaglandins were found to be common to several forms of P-450. Several new metabolites were detected, isolated and identified.

Purified cytochromes P-450 were classified by measuring spectral and immunological properties. In the latter case, 16 MABs and 4 polyclonal antisera to different P-450s were used. The data strongly suggests that the P-450 superfamily consists of five or six subfamilies. P-450 members belonging to a certain subfamily had structural and spectral similarities to one another. The data suggests that a common type of P-450 exists in liver, kidney and/or lung microsomes and there are several highly similar forms of liver microsomal P-450 in rats and rabbits.

Cross-reactivities between two forms of purified P-450 and 16 MABs were measured in 27 different solvent conditions in an RIA. In several cases, such as low ionic strength, high and low pH, and denaturation treatments, cross-reactivities greatly increased or decreased. These changes imply conformation changes on protein surface. These results were further reinforced by experiments with MAB inhibition of catalytic activities, such as benzo(a)pyrene hydroxylation and benzphetamine N-demethylation.

Fifteen MABs were used to study the epitope topography of P-450 on the microsomal membrane. The results indicated that at least seven kinds of epitopes are located on the cytoplasmic side of the microsomal membrane. This conclusion was also confirmed by experiments with inhibition of catalytic activities by MABs.

The null MAB(1-36-1) was linked to agarose to yield an immunoabsorbent which was used for immunopurification of P-450c from liver microsomes of NF-treated rats. P-450c having a normal heme spectra was eluted with a high concentration of detergent and salt.

The immunoabsorbent was linked to P-450c to yield a P-450-bioreactor. This bioreactor was incubated with substrate(7-ethoxycoumarin) and hydrogen peroxide as oxygen donor. This bioreactor produced metabolite at a very high turnover for the first several minutes, but P-450c was rapidly denatured.

Publications:

None.

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05475-01 LMC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Structure and Characterization of Ethanol-Induced P-450		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Frank J. Gonzalez	Sr. Staff Fellow LMC NCI
Others:	Byung J. Song Harry V. Gelboin Jullia Pastewka Sang S. Park	Visiting Fellow LMC NCI Chief LMC NCI Chemist LMC NCI Expert LMC NCI
COOPERATING UNITS (if any) New Jersey Medical School, Department of Biochemistry (Chung S. Yang)		
LAB/BRANCH Laboratory of Molecular Carcinogenesis		
SECTION Metabolic Control Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> Certain forms of P-450 have a high catalytic activity toward carcinogens. P-450s can either metabolically activate procarcinogens to electrophilic metabolites or inactivate these compounds by hydroxylation followed by another enzymatic conjugation reaction. The levels and types of P-450s in the liver and other tissues therefore could determine the fate of ingested carcinogens. We have been studying the enzyme P-450j which has a high activity toward the potent carcinogen N-nitrosodimethylamine. The demethylation reaction carried out by this P-450 leads to highly reactive intermediates that can bind and mutate DNA. A monoclonal antibody against rat P-450j was used to isolate rat and human P-450j cDNA clones. These cDNAs were sequenced and their nucleotide and deduced amino acid sequences were 78 and 80% similar, respectively. The rat P-450j monoclonal antibody and cDNA clones were utilized as probes to determine the mechanism whereby P-450j is regulated by ethanol, acetone, and the alcohol dehydrogenase inhibitors, 7-methyl-pyrazole and pyrazole. A unique translational control of P-450j by these compounds was uncovered. The fourfold elevation in P-450j protein in rat microsomes by these agents is not accompanied by an increase in its mRNA. In contrast to this post-transcriptional regulation, P-450j is increased during the first few days after birth through a massive increase in gene transcription rate. </p>		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on This Project:

Frank J. Gonzalez	Sr. Staff Fellow	LMC	NCI
Bjung J. Song	Visiting Fellow	LMC	NCI
Harry V. Gelboin	Chief	LMC	NCI
Sang S. Park	Sr. Staff Fellow	LMC	NCI
Jullia Pastewka	Chemist	LMC	NCI

Objectives:

(1) Isolate and sequence cDNA clones for rat and human P-450j, (2) isolate and sequence genomic clones for rat and human P-450j, (3) determine the mechanism of regulation of P450j during development and by inducing agents, (4) examine individual variations in expression of P-450j in human tissue samples, and (5) express P-450j in tissue culture and determine the role of this enzyme in cell transformation by carcinogens.

Methods Employed:

Libraries of cDNA clones are prepared in the expression vector λ gt11. This vector produces protein from cloned inserts in *E. coli* and this protein can be detected by use of specific antibodies. DNA inserts from isolated clones are sequenced by use of the m13 phage cloning and dideoxynucleotide sequencing strategy. Messenger RNA for P-450j will be analyzed by Northern blots and slot blots. Genomic DNA will be characterized by Southern-blot analysis.

Major Findings:

Human and rat P-450 cDNAs were isolated and sequenced. The protein sequence of human P-450j is 80% similar to rat P-450j. Rat P-450j shows about 48% similarity to the major phenobarbital-induced P-450, P-450e, but shows less than 30% similarity to 3-methylcholanthrene-induced P-450c.

Rat P-450j is absent in newborn rats but increases dramatically within two days after birth. This developmental increase is due to transcriptional activation of the P-450j gene. In adult rats, P-450j is elevated fourfold when animals are given acetone or ethanol in their drinking water. This increase is not accompanied by an elevation in P-450j mRNA, suggesting that P-450j is post-transcriptionally controlled.

Publications:

Song, B. J., Gelboin, H. V., Park, S. S., Yang, C. S. and Gonzalez, F. J.: Complementary DNA and protein sequence of rat and human ethanol-induced P-450: transcriptional and post-transcriptional regulation of the rat enzyme. J. Biol. Chem. (In Press).

Patents:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CP05476-01 LMC
PERIOD COVERED October 1, 1985 to September 30, 1986		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Developmental Regulation of P-450s		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Frank J. Gonzalez	Sr. Staff Fellow LMC NCI
Others:	Harry V. Gelboin Byung J. Song Morio Umeno Jullia Pastewka	Chief LMC NCI Visiting Fellow LMC NCI Visiting Fellow LMC NCI Chemist LMC NCI
COOPERATING UNITS (if any) Argonne National Laboratory, Argonne, IL (James P. Hardwick)		
LAB/BRANCH Laboratory of Molecular Carcinogenesis		
SECTION Metabolic Control Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Certain P-450s are developmentally regulated. These include testosterone-16 α -hydroxylase, an adult male specific P-450, and testosterone-15 α -hydroxylase, an adult female specific P-450. These two enzymes increase during rat development and their increase is mediated through blood androgen levels and pulsatile levels of growth hormone. We have been analyzing two classes of P-450s: one class of P-450, including P-450f and P-450PB-1, have no known steroid hydroxylase activities. These P-450s increase during development in both males and females and reach maximal levels at rat maturity. This increase is independent of androgens. We cloned and sequenced cDNAs for these enzymes and found that they share 75% amino acid homology. The cDNA probes were used to show that these P-450s increase during development as a result of transcriptional activation of their respective genes. These P-450s are probably linked and are coordinately regulated. The cDNA of a P-450 that is induced by steroids was cloned and sequenced. This P-450, designated P-450PCN1 (PCN, pregnenolone-16 α -carbonitrile), was found to be absent in noninduced rats and induced by PCN and phenobarbital. Another P-450 cDNA was isolated based on its sequence homology with P-450PCN1 and designated P-450PCN2. P-450PCN2 has 90% amino acid sequence similarity with P-450PCN1. This homology is present in three distinct regions of 99% to 100% similarity surrounded by regions of 80 to 85% similarity. These data suggest that gene conversion events have played a role in evolution of this P-450 family. Interestingly, P-450PCN2 is a developmentally regulated P-450. Its level increases in young male and female rats but declines in female rats at puberty and continues to increase in male rats, reaching maximal levels at maturity. In contrast to P-450PCN1, P-450PCN2 is not induced by steroids such as PCN and dexamethasone. Similar to P-450PCN1, P-450PCN2 is induced by phenobarbital. Induction and developmental studies suggest that both P-450s possess testosterone-6 β hydroxylase activity.		

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on This Project:

Frank J. Gonzalez	Sr. Staff Fellow	LMC	NCI
Harry V. Gelboin	Chief	LMC	NCI
Byung Y. Song	Visiting Fellow	LMC	NCI
Morio Umeno	Visiting Fellow	LMC	NCI
Jullia Pastewka	Chemist	LMC	NCI

Objectives:

(1) Isolate P-450s responsible for steroid metabolism and produce antibodies, (2) isolate and characterize their cDNAs, (3) isolate and characterize genomic clones, (4) study the mechanism of P-450 gene activation during development, and (5) determine the role of these developmentally regulated P-450s in physiological metabolism and carcinogen metabolism.

Major Findings:

P-450f and P-450PB-1 are developmentally regulated in both male and female rats. cDNAs for these P-450s were isolated and sequenced and their deduced amino acid sequences found to be 75% similar.

The mechanism of regulation of these P-450s during development was studied. Both P-450s are absent in newborn rats and their levels begin to increase at two weeks of age and reach maximal levels between 4 and 12 weeks of age. This increase was due to transcriptional activation of the P-450f and P-450PB-1 genes. These P-450s represent a new developmentally regulated family of P-450s.

Another P-450, P-450PCN2 (PCN, pregnenolone 16 α -carbonitrile), was discovered through cloning its cDNA. This P-450 has not been previously isolated. Sequence of P-450PCN2 cDNA revealed that it shares 89% amino acid similarity with P-450PCN1. This latter P-450 is absent in noninduced rats but is readily induced by steroids such as dexamethasone and PCN. In contrast, P-450PCN2 is not induced by steroids. Both P-450PCN1 and P-450PCN2 are induced by phenobarbital. P-450PCN2 is a male-specific enzyme. Levels of this P-450 increase in both males and females within two weeks of age. In males, P-450PCN2 continues to increase to maximal levels at maturity while in females this P-450 decreases and is absent in adults. Antibody inhibition studies suggest that both P-450PCN1 and P-450PCN2 possess testosterone-6 β -hydroxylase activity.

Publications:

Gonzalez, F. J., Kimura, S., Song, B. J., Pastewka, J., Gelboin, H. V., and Hardwick, J. P.: Sequence of two P-450s transcriptionally regulated during development: An R.dre.1 sequence occupies the complete 3' untranslated region of a liver mRNA. J. Biol. Chem. (In Press).

Gonzalez, F. J., Song, B. J. and Hardwick, J. P.: Gene conversion and differential regulation in the pregnenolone-16 α -carbonile-inducible P-450 gene family. Mol. Cell. Biol. (In Press).

Patents:

None

ANNUAL REPORT OF
THE CHEMICAL AND PHYSICAL CARCINOGENESIS BRANCH
THE CHEMICAL AND PHYSICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1985 through September 30, 1986

The Chemical and Physical Carcinogenesis Branch (CPCB) plans, coordinates, and administers a national extramural program of basic and applied research consisting of grants and contracts, collectively concerned with the occurrence and the inhibition of cancer, caused or promoted by chemical or physical agents acting separately or together, or in combination with biological agents; plans, organizes, and conducts meetings of scientists and otherwise maintains contacts with scientists-at-large, to identify and evaluate new and emergent research in, and related to, the fields of chemical and physical carcinogenesis; provides a broad spectrum of information, advice, and consultation to scientists and to institutional science management officials, relative to the National Institutes of Health (NIH) and National Cancer Institute (NCI) funding and scientific review policies and procedures, preparation of grant applications, and choice of funding instrument, based on individual need; plans, develops, maintains, and allocates research resources necessary for the support of carcinogenesis research of high programmatic interest; and provides NCI management with recommendations concerning funding needs, priorities, and strategies relative to the support of chemical and physical carcinogenesis research, consistent with the current state of development of individual research elements and the promise of potential, new initiatives.

Research and related activities supported under this program bear upon a broad range of subject matter areas, with principal emphasis on environmental carcinogenesis, mechanisms of action of chemical and physical carcinogens; the role of DNA damage and repair in carcinogenesis; properties of cells transformed by chemical and physical agents; inter- and intraspecies comparisons in the response to carcinogen exposure; the role of tumor promoters, hormones, and other cofactors in cancer causation; experimental approaches to the inhibition of carcinogenesis; the role of diet and nutrition in carcinogenesis; the role of tobacco products and smoking in carcinogenesis; and in vitro carcinogenesis studies on human and other mammalian cells, tissues, and subcellular fractions. The program also supports the synthesis, acquisition, and distribution of a considerable spectrum of chemical standards, critically needed in the field of carcinogenesis research.

Grants and contracts administered by the staff of this Branch support six complementary categories of chemical and physical carcinogenesis research and associated resources: Biological and Chemical Prevention, Carcinogenesis Mechanisms, Diet and Nutrition, Molecular Carcinogenesis, Smoking and Health, and Research Resources.

The Biological and Chemical Prevention component is concerned with the experimental inhibition of carcinogenesis caused by chemical, physical, and biological agents. Efforts are devoted to the identification, development, and testing (both in vitro and in vivo) of agents intended to inhibit carcinogenesis. Areas of prime interest include mechanisms of action of candidate preventive agents, binding proteins and receptors, structure-function relationships, and the experimental use of combinations of preventive agents.

The Carcinogenesis Mechanisms category relates to the absorption and body distribution of carcinogens; metabolism, activation, and inactivation of carcinogens; identification of proximate and ultimate carcinogenic forms; molecular structure-carcinogenicity relationships; carcinogen-mutagen relationships; isolation, identification, and synthesis of suspect carcinogens and their metabolites; factors which alter carcinogen activity; the characterization of carcinogen metabolizing enzymes; and the role of hormones in carcinogenesis.

The Diet and Nutrition category supports basic studies on the carcinogenic and anticarcinogenic effects of diet and specific nutrients in animal systems and human cells in vitro.

The Molecular Carcinogenesis component focuses on changes in biological macromolecules and in cell functions as a result of carcinogen exposure; DNA damage and repair following exposure to carcinogens; identification of biochemical and molecular markers and properties of cells transformed by carcinogens; the development of analytical procedures for the identification and quantitation of carcinogens present in biological specimens; interspecies comparisons in carcinogenesis; the role of tumor promoters and the mechanism of tumor promotion in carcinogenesis; and studies on the genetics and mechanism of cell transformation and of the genetics and regulation of enzymes characteristically associated with the carcinogenesis process.

The Smoking and Health category supports studies on the toxicology and pharmacology of smoking and tobacco-related exposures. Both grant and contract mechanisms are used to support these activities.

The Research Resources component, supported solely by contract, is principally concerned with the synthesis and distribution of selected chemical carcinogens and certain of their metabolites, with particular reference to polynuclear hydrocarbon carcinogens, their metabolic intermediates, and analogous heterosubstituted compounds, as well as the synthesis and distribution of retinoids including radio-labeled forms.

This fiscal year has proved to be a busy and scientifically successful year for the Chemical and Physical Carcinogenesis Branch. The first year implementation of the Graham-Rudman-Hollings Act posed an additional administrative burden for the Branch both in budgetary reductions and increased fiscal scrutiny of all awards and initiatives. The Scientific Community has shared both in the anxiety over the budgetary ramifications of the Act but also in the fact that all new (T-1) and recompeting (T-2) grants were reduced by 9% from the study section recommended amount. Noncompeting (T-5) awards were reduced by 5%. This method of reduction was, however, quite reasonably received by grantees who realized that all would be sharing in carrying the budget burden. The Branch took some reductions in its own operating budget as well.

This has been an active year for new and recompeting scientific initiatives. During this year the Branch recompeted all major contract initiatives of the Resources component, "The Chemical Carcinogen Repository," the "Synthesis of PAH Derivatives," and has initiated the recompetition for the "Synthesis of Selected Chemical Carcinogens." The Smoking and Health component sought and obtained concept approval for the renewal of an Interagency Agreement with the Department of Energy at the Oak Ridge National Laboratory on "Collection, Separation and Elucidation of the Components of Cigarette Smoke." This followed the results of a postive site visit to Oak Ridge during the Winter.

The Branch issued two Program Announcements in FY 1986. An announcement on "The Biological Role of Exocyclic Nucleic Acid Derivatives in Carcinogenesis" was issued in January from the Molecular Carcinogenesis Component. This type of initiative encourages the submission of grant applications dealing with a specified topic for assignment to the Branch. It specifically differs from an RFA in that there are no set-aside funds, and no special study section for unified review is offered. This announcement seeks applications on basic mechanistic studies focused on determining the formation, repair and relevance to mutagenesis and carcinogenesis of exocyclic nucleic acid derivatives. The compounds of interest which are known or are likely to form exocyclic nucleic acid derivatives include: vinyl halides (vinyl chloride, vinyl bromide), alkyl carbamates (ethyl and vinyl carbamate), halonitrosoureas (BCNU, CCNU), monofunctional unsaturated aldehydes (acrolein, crotonaldehyde), bifunctional aldehydes (glyoxal, malonaldehyde, glycidaldehyde), beta-propiolactone, acrylonitrile, N-nitrosopyrrolidine and related cyclic nitrosamines, and some halogenated ethers and aldehydes (chloro- and bromoacetaldehyde). Examples of important areas of research emphasis include the following: 1) the identification and quantitation of adducts which may be responsible for the carcinogenicity of the test compound in animals, the transformation of cells in culture, or the mutagenicity of the compound in cells in culture or in other test systems; 2) the formation and repair of exocyclic adducts in animals, cells in culture, or other test organisms relevant to carcinogenicity, transformation and mutagenicity studies; and 3) the mechanism of mutagenesis or carcinogenesis by exocyclic nucleic acid adducts, other adducts of biological interest or cross-links which may be formed by the above-mentioned compounds. It is also recognized that there will be a need to develop more sensitive methods to analyze and quantitate the many possible adducts and to detect them in DNA from cells exposed to the chosen compounds. A desired sensitive method, not widely available, is an immunoassay using monoclonal antibodies to the chosen exocyclic adduct or other relevant adduct. It is suggested that support for the development of such monoclonal antibodies may, in some cases, be appropriate for the SBIR grant/contract program as well as the traditional (R01) grant.

In February, the DCE Board of Scientific Counselors approved a concept for another Program Announcement, released during the Summer of 1986, to encourage basic mechanistic studies on the role of omega-3 polyunsaturated fatty acids in cancer prevention. Among the areas of particular interest are: (1) anticarcinogenesis studies in various organ systems, particularly those organ systems in which the type and level of fat have been shown to play a role; (2) determination of whether efficacy is obtained during the initiation period by modifying the susceptibility of the host to early events, or whether these fatty acids modulate the carcinogenic response in the post-initiation period, or both, and including determination of efficacy over the lifetime of the animal; (3) pharmacokinetic studies on the absorption, distribution, metabolism and excretion of these fatty acids, including such studies performed under the experimental conditions demonstrating cancer prevention; (4) studies on toxicology of the agents, including lifetime administration studies under defined dietary conditions in several species of animals; (5) comparative metabolic studies in human vs animal systems; (6) in-depth studies of mechanisms of action, especially as related to conditions known or demonstrating anticarcinogenic efficacy. It is particularly desired that mechanism studies on anticarcinogenesis be reflective of the current state-of-the-art in molecular and cellular carcinogenesis, experimental pathology, immunology, endocrinology, cocarcinogenesis and tumor promotion. Program Projects and/or consortial arrangements under traditional R01 grants are encouraged where

collaborating expertise, special facilities and equipment are deemed necessary to approach and carry out these investigations.

This initiative stems from published observations that cancer risk is higher among people who consume diets high in fat and low in fiber, vegetables and micro-nutrients. Additionally, recent studies have demonstrated that not only the amount of fat, but the composition and type of fat consumed have a significant influence on the development of cancer.

Fats containing polyunsaturated fatty acids (PUFA) of the omega-6 family are apparently more favorable to the growth of tumor cells. The PUFA generally consumed are derived from vegetable oils which contain high levels of linoleic acid. Experiments with laboratory animals have demonstrated that dietary linoleic acid favors the growth of tumor cells. The mechanism(s) of fatty acid-enhanced tumorigenesis and tumor growth are not well defined. Possible mechanisms include the fact that polyunsaturated fatty acids can easily undergo oxidation to yield a variety of mutagens, promoters, and carcinogens, such as fatty acid hydroperoxides, endoperoxides, enals, aldehydes, alkoxy, and hydroperoxy radicals which promote the growth of cancer cells. In addition, polyunsaturated fatty acids like linoleic acid give rise to arachidonic acid when elongated and desaturated. Arachidonic acid is the precursor for biologically active prostaglandins, such as prostaglandin E_2 (PGE_2). PGE_2 exerts suppressive action on immunological cells, which is postulated to enable tumor cells to escape the immunosurveillance of the body and metastasize and proliferate. There is strong evidence that omega-6 PUFA are conducive to promotion of cancer by virtue of their ability to elicit production of immunosuppressive prostaglandins.

It is not feasible to eliminate PUFA completely from the human diet to reduce the risk of cancer because these PUFA are needed for normal biochemical functions and the maintenance of normal health. Furthermore, there is widespread advocacy for increased consumption of omega-6 PUFA (vegetable oils) to improve serum lipid levels and reduce coronary heart disease.

Ideally, we need a source of dietary PUFA that would exert beneficial effects on coronary heart disease while suppressing PGE_2 production, thereby decreasing cancer. The omega-3 PUFA which occur in fish oils, particularly from fish that live in deep, cold waters, may serve that function. Fish oils extracted from mackerel, bluefish, herring, and menhaden, for instance, have low levels of omega-6 fatty acids, but contain high levels of omega-3 PUFA, such as eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6). Epidemiological studies with Greenland Eskimos, Japanese, and Icelanders indicate that the population consuming seafood regularly are less prone to coronary heart diseases, atherosclerosis, hypertension, and cancer. However, changes in their food habits to western style diets is correlated with increased mortality rates from cancer. Recent studies have demonstrated that diets containing these omega-3 fatty acids effectively retard the growth of tumor cells in animal models. Despite these observations, the mechanism underlying the relationship between dietary fat and cancer is not well understood.

It has been generally observed that the high serum levels of PGE_2 derived from omega-6 PUFA are conducive to the growth of tumor cells and there is a good correlation between the levels of prostaglandin E_2 and tumor growth in experimental animals. Fish oil-enriched diets decrease the formation of PGE_2 , and this coincides with the retarded growth of tumor cells. In this respect, monocyte-macrophages are important relevant cells which are the major producers of PGE_2 .

Massive invasions of macrophages have been observed in tumors. Because of the presence of high levels of PGE₂, these macrophages do not function in their normal capacity as cytotoxic cells against tumors. However, by reducing the local concentrations of PGE₂, the suppressive action of PGE₂ on macrophage function could be relieved. Dietary intervention with fish oils may provide such an approach. It has been demonstrated that macrophages can effectively take up omega-3 fatty acids from dietary sources. This reduces cellular arachidonic acid levels, and subsequently decreases their capacity to synthesize PGE₂. Thus, the overall levels of PGE₂ can be decreased by dietary omega-3 fatty acids, and thereby relieve the inhibition of the phagocytic activity of the macrophages. This could retard the growth of tumor cells. In addition, it has been observed that omega-3 fatty acid enrichment also enhances arginase production in macrophages, and this enzyme exerts cytolytic action on tumors. Thus, dietary omega-3 fatty acids could significantly retard the growth of tumor cells without affecting the normal functions of macrophages. Significantly, the omega-3 PUFA of fish oils may, by inhibiting cyclooxygenase and reducing PGE₂ synthesis, divert the arachidonic acid into the lipoxygenase pathway which produces compounds such as hydroxyeicosatetraenoic acid (HETE) and leukotrienes (LT), e.g., LTB₄. These compounds are chemotactic agents for macrophages and other immunological cells, which function in the control of tumors. Most significantly, HETE inhibits the growth of tumor cells.

Of especial interest have been recent studies directed at determining the potential effectiveness of dietary fish oils in cancer prevention in animals. Although results are only preliminary at this time, high levels of dietary fish oil (menhaden oil) appear to inhibit or retard the development of MNU-induced mammary tumors, and the development of azaserine-induced putative preneoplastic lesions of the pancreas in rodents. In addition, fish oils contain high levels of retinoids (Vitamin A) which can act as antineoplastic agents. Furthermore, omega-3 fatty acids can effectively reduce plasma cholesterol levels, and lower cholesterol levels may reduce the risk of cancer, whatever may be the mechanisms. Hence, omega-3 PUFA may act via a number of mechanisms. These observations suggest that fish oils and/or seafood-based diets may provide an effective non-invasive dietary intervention approach for reducing the risk of tumor growth and cancer.

Grants that are awarded as a result of the Omega-3 Fatty Acid Program Announcement will be assigned to the Biological and Chemical Prevention Component.

During the Summer of 1985, the Biological and Chemical Prevention Program issued an RFA for National Collaborative Chemoprevention Projects (NCCPs) which are conceived as new approaches to cancer prevention in order to: acquire basic knowledge in significant biological systems for carcinogenesis/anticarcinogenesis, derive new insights into practical means for chemoprevention of the carcinogenic process, and rapidly translate these understandings into new chemopreventive entities with known ranges of efficacy and defined pharmacologic/toxicologic properties.

The three NCCPs, which were awarded late in FY 1986 as a result of this announcement, were funded as cooperative agreements. The cooperative agreement is an assistance mechanism in which the Government component (NIH, NCI) making the award anticipates substantial programmatic involvement with the recipient during performance of the planned activity. Each NCCP could consist of a number of laboratory research programs representing diverse scientific disciplines and expertise, such as experimental carcinogenesis, pharmacology, toxicology,

medicinal and organic chemistry, molecular and cellular biology, biochemistry, immunology, and pathology. Scientists in a given Project could derive from any combination of the academic, non-profit, and for-profit communities. Scientists in an NCCP could also be drawn from a single organization possessing necessary diversity and indepth expertise to accomplish Project objectives. Each Project is envisioned to consist of a Project Director, Program Leaders in several broad scientific disciplines, and an NCI Coordinator. The Project Director has the responsibility for organizing the Project, assembling the multidisciplinary group of Program Leaders, preparing the cooperative agreement application, and serving as Principal Investigator. This individual provides scientific and administrative leadership and, in addition, is expected to provide a laboratory program. A high degree of interaction and focus are expected in Project efforts.

The overall objective of the first NCCP grant (1 U01 CA 43311-01) at the University of Texas Health Sciences Center is to explain why dehydroisoandrosterone is extraordinarily beneficial in the prevention and amelioration of a variety of disorders, principally neoplasia, autoimmune disorders, diabetes, and obesity which are currently "incomprehensible."

The specific approaches are to identify its effects on hepatic intermediary metabolism, principally fatty acid including arachidonic acid metabolism, gluconeogenesis and cholesterol synthesis, the conversion of precarcinogens to reactive intermediates by Phase I and II enzymes, and effects on the immune system. This provides an extensive overview of a number of possible mechanisms by which DHA may exert its effects.

Dr. MacDonald will direct the project which is largely centered in Dallas, Texas. The work will be divided into four main programs:

Program 1, led by Dr. MacDonald, will evaluate the metabolism of DHA. This is a critical portion of the overall project since an understanding of whether DHA or some of its metabolites are the active components of the pleiotrophic responses to DHA may lead to the development of even more effective agents. In addition, some specific ideas of how DHA may influence fatty acid metabolism, in particular, and intermediary metabolism, in general, will be explored.

Program 2, directed by Dr. Prough, will take place at the University of Louisville. The main objective of this program is, having made the assumption that metabolic activation of precarcinogens is critical to the action of DHA, to purify specific P-450 enzymes. Only in later years will the effects of this modulation of BP metabolism be considered in vivo with respect to DNA adduct formation. Additional enzymes involved in xenobiotic metabolism will be studied with a variety of carcinogens. Towards the end of the project some thought will be given to the possible effects of promotion.

Program 3, directed by Dr. Bennett at the University of Texas Southwestern Medical School, will investigate possible effects of DHA on a variety of aspects of the immune system. Initially they will evaluate the effects of DHA feeding on natural killer and cytotoxic cell functions including cytotoxic lymphocyte production, antibody formation, and resistance to infection by mycoplasma pulmonis. Studies will also be performed on the effects of DHA feeding on irradiation-induced lymphomas, immunosuppression by BP painting, and MTV-induced breast cancer.

Program 4, directed by Dr. Cottam at Dallas, has interest in fatty acid metabolism. The hypothesis is that DHA may cause local deficiencies of

arachidonic acid. To evaluate this, fatty acid compositions of the plasma, kidney, liver, and macrophages will be compared from mice fed DHA and controls. In common with Program 3, the immune system will be considered and macrophages evaluated for phagocytic activity, antigen presentation to T-cells, metabolism of arachidonic acid to prostanoids as well as their fatty acid compositions as described above. In addition, sera levels will be compared in DHA and control animals for tumor necrosis factor which, being identical to cachectin, induces hyperglycemia, decreases lipogenic activity and could explain the dual antitumor and anti-obesity effects of DHA feeding.

The second NCCP (1 U01 CA 43277-01) will be directed from the University of California School of Medicine in Los Angeles with collaborators at the Riverside campus. The objective of this grant is the synthesis of new vitamin D derivatives that will combine antileukemic effects with low capability to stimulate hypercalcemia. Recent studies have demonstrated that $1,25(\text{OH})_2\text{D}_3$, one of the most active derivatives of vitamin D, in addition to its effects on bone and intestinal epithelium that regulate the homeostasis of calcium and phosphorus, also induces differentiation of leukemic cell lines. This compound binds to receptors present on hemopoietic stem cells and also can be metabolized by these cells. The ability of $1,25(\text{OH})_2\text{D}_3$ to regulate differentiation of marrow stem cell derivatives (normal and neoplastic) prompted investigations for the use of this compound for inhibiting growth (by stimulation of differentiation) of myeloid leukemias. These studies, however, could not be meaningfully concluded because it was found that administration of $1,25(\text{OH})_2\text{D}_3$, even at relatively low levels, was accompanied by severe hypercalcemia. This study brings together a team composed of a clinical expert with experience on effects of $1,25(\text{OH})_2\text{D}_3$ on leukemia and studies of leukemia in general (Dr. Koeffler), two vitamin D biochemists with excellent track records (Drs. Norman and Henry), and a well-recognized vitamin D chemist (Dr. Okamura). The objective of the combined effort is the synthesis of new vitamin D derivatives that will manifest only the desirable effect of antileukemic action without the hypercalcemic effect. The authors make the assumption that the receptors for $1,25(\text{OH})_2\text{D}_3$ in different tissues will differ somewhat in their requirements for optimal ligand interaction. If this is true, several new vitamin D derivatives will be synthesized that would be active preferentially on the marrow cell receptor and less so on the receptors of tissues, such as bone and intestine that are involved with the production of hypercalcemia.

Dr. Koeffler (at UCLA) is the director for the project. He will also evaluate the new vitamin D derivatives in several in vitro and in vivo systems of leukemic cell differentiation. Dr. Okamura, at Riverside, is the chemist responsible for the synthesis of the new derivatives. He proposes specific areas where the basic molecule can be modified with the most likely chance of achieving the desired properties. Dr. Norman, also at Riverside, will use techniques established in his laboratory for the isolation and characterization of the $1,25(\text{OH})_2\text{D}_3$ receptor from the HL-60 cell line. For this purpose he will employ the same techniques that were used for the isolation of the chick intestinal receptor for the same molecule in his laboratory. In addition, Dr. Norman will study the binding of the new derivatives to the $1,25(\text{OH})_2\text{D}_3$ receptor of HL-60 and chicken intestinal cells. Dr. Henry will test the effect of the new compounds on the activity of 1, α hydroxylase in chicken kidney cells.

The objective of the third NCCP at the University of Minnesota, with collaboration from IIT Research Institute, is to obtain compounds that will trap direct-acting carcinogens within the lumen of the gastrointestinal tract and prevent them from attacking host tissues. Two major attributes required of the compounds are

efficacy as trapping agents and retention within the gastrointestinal tract. This latter feature is important in providing effective concentrations throughout the entire alimentary tract and potentially providing safe compounds in terms of lack of toxicity. The specific aims of the proposal are as follows: (1) to identify candidate compounds and provide these for testing, (2) to determine the capacity of the candidate compounds to react with a diverse group of direct-acting carcinogens in vitro and also with fecapentaene, (3) to determine retention of the test compounds within the gastrointestinal tract, (4) to determine the capacity of test compounds to trap direct-acting carcinogens within the lumen of the gastrointestinal tract in vivo, (5) to determine the capacity of test compounds to inhibit carcinogen-induced neoplasia resulting from administration of direct-acting carcinogens, and (6) to determine toxicity of effective test compounds.

The NCCP consists of an administrative unit (Dr. L. Wattenberg) and laboratories at two institutions. One of these is the University of Minnesota, Department of Laboratory Medicine and Pathology (Dr. L. Wattenberg) and Department of Chemistry (Dr. G. Barany). Dr. Richard Moon at the IIT Research Institute will collaborate with Dr. Wattenberg in the in vivo aspects of this NCCP.

The Board of Scientific Counselors gave concept approval to two related initiatives on neoplasia in fish and shellfish in 1985 (May). Both of the projects are progressing well. The first concept was for an RFA for "Studies on the Etiology of Neoplasia in Poikilothermic, Aquatic Animals: Finfish and Shellfish." The RFA was issued on January 31, 1986 and closed 90 days later on May 1, 1986. It was reviewed on July 15-16 and subsequently considered by a mail ballot to the National Cancer Advisory Board on August 15. Award of the eligible grants is scheduled for FY 1986 prior to October 1. The urgency is due in part to the fact that multiple agencies are contributing to this effort with funds allocated for FY 1986. The RFA was initially delayed due to uncertainties in the availability of funding between agencies, but the award will commit up to \$500,000 of NCI/DCE funds together with \$200,000 from the National Institute of Environmental Health Sciences and \$100,000 from the Department of The Army, Medical Research and Development Command. Six to eight awards are planned. At the time of this writing there are 54 grant applications competing for these funds. The level of interest in this RFA was heightened in part by a wide dissemination of the RFA through a special mailing to Marine Laboratories and to those publishing in this field in addition to the traditional listing in the "NIH Guide to Grants and Contracts."

Consistent with the title of this proposed RFA are a broad spectrum of studies that would greatly facilitate our understanding of the etiology of neoplasia in finfish and shellfish. Listed below are some commonly identified needs which expressed the spectrum of studies of interest but were not intended as a comprehensive list of possibilities (it was up to the applicant to determine the scope and objective of the studies proposed):

- a) Evaluation of the similarity of metabolic function in procarcinogen activation among different species of invertebrates and/or vertebrates in regard to Phase I and Phase II reactions. Assessment of the role of fish hepatocytes in metabolism of procarcinogens. Studies on bioavailability and transfer of xenobiotics and their metabolites from invertebrates to fish and from invertebrates and fish to mammals.

- b) Effects of environmental and physiological variables of water temperature, age, sex and gonadal development on bioavailability and metabolism of xenobiotics.
- c) Development of in vitro culture systems for normal and neoplastic cells from invertebrates and vertebrates and analysis of adducts to macromolecules of environmentally relevant xenobiotic metabolites.
- d) Studies on chemical/chemical and chemical/viral interaction in the etiology of aquatic animal neoplasms and the identification of oncogenes in invertebrate and vertebrate species.
- e) Analysis of DNA repair capacity, mitotic index, sister chromatid exchange, cell cycle time, and enzyme pathways for xenobiotic metabolism under various temperature conditions in poikilothermic aquatic animals and determination of the relationship to the persistence of genetic lesions that might lead to tumorigenesis.
- f) Studies of factors involved in promotion or progression of a tumor in aquatic species. Assessment of transplantability of neoplasms.
- g) The effect of chemical pollutants on the immune response in aquatic animals and the role of the immune system in aquatic animal neoplasia.
- h) Expansion of experimental oncology databases on various promising fish species as carcinogen assay subjects. Tumor induction studies with chemical agents utilizing species such as the rainbow trout, the medaka or Japanese killifish, the guppy, the zebrafish, the sheepshead minnow, or other established models. Besides tumor induction, studies might include the further development of data on potency of carcinogenic agents in fishes, utilizing fish molecular, cellular, and tissue responses with screening endpoints such as unscheduled DNA synthesis, liver enzyme induction, chromosomal aberrations, sister chromatid exchange, or detection of altered foci in such target tissues as liver.

Essentially all of these possibilities were covered in the 54 applications received.

The other related initiative this year was progress on the development of an Atlas on Neoplasms and Related Disorders in Fishes. The purpose of the Atlas will be to present high quality photomicrographs and/or electron micrographs of all the known neoplastic histotypes in fishes, together with the preferred classifying terms and synonyms that have been applied in the past. Where homologous neoplasms in different species have been seen to differ histologically, illustrations of these differences would be included (e.g., nephroblastomas in rainbow trout, American eel, and striped bass have been observed to have marked histological differences). Each histotype portrayed would be accompanied by a relatively brief text citing the historical development of relevant information, listing any known etiological

behavior of the neoplastic type. A list of the best available literature references would be appended to each section.

The Atlas on Neoplasms and Related Disorders in Fishes will be a highly visible publication that will serve to establish the standards of histopathological nomenclature in fishes and will clarify the terminology and significance of the conditions in fish histopathology. In addition, it will become a valuable reference tool and the standard of nomenclature in this field of study.

During the last year, the Branch has had two successful applicants receive the new outstanding investigator awards of 7 years' duration. The recipients were Dr. Bruce Ames (5 R35 CA 39910-02) and Dr. Lawrence Loeb (5 R35 CA 39903-02). Their grants are assigned to the Molecular Carcinogenesis component and will be reported on there.

The Branch was successful in nominating four other research grant applicants for the even newer MERIT awards. These investigators were Dr. James P. Whitlock (2 R01 CA 32786-04) Stanford University, Dr. Thomas A. Brasitus (2 R01 CA 36745-04) Michael Reese Hospital and Medical Center, Dr. William Baird (2 R01 CA 28825-07) Purdue University, and Dr. Joachim G. Liehr (2 R01 CA 43233-02) University of Texas Medical Branch.

A summary of the number of grants, contracts, and associated funding relative to each of the above categories and to the Chemical and Physical Carcinogenesis Branch, as a whole, follows. Table I focuses on mechanisms of support of extramural research and related activities in the area of Chemical and Physical Carcinogenesis. Table II provides an estimate of grant and contract support, respectively, in each of the six Branch components as described above.

TABLE I
CHEMICAL AND PHYSICAL CARCINOGENESIS BRANCH
(Extramural Activities - FY 1986 - Estimated)

	No. of Contracts/Grants	\$ (Millions)
Research Contracts	6	1.01
Research Grants	485	57.33
Traditional Research Grants (R01) (412 grants; \$42.02 Million)		
Conference Grants (R13) (9 Grants; \$0.04 million)		
New Investigator Research Grants (R23) (18 Grants; \$0.65 million)		
Program Project Grants (P01) (9 grants; \$8.24 million)		
Cooperative Agreements (U01) (3 grants; \$1.47 million)		
Small Business Grants (R43/R44) (0 grants; \$0.00 million)		
Outstanding Investigator Grants (R35) (2 grants; \$0.79 million)		
RFAs (R01) (28 grants; \$3.52 million)		
MERIT Awards (R37) (4 grants; \$0.60)		
Research Resource Contracts	9	1.28
TOTAL	500	59.62

TABLE II
CHEMICAL AND PHYSICAL CARCINOGENESIS BRANCH
(Contracts and Grants Active During FY 1986)

FY 1986				
	CONTRACTS		GRANTS	
	<u>No. of Contracts</u>	<u>\$ (Millions)</u>	<u>No. of Grants</u>	<u>\$ (Millions)</u>
Biological & Chemical Prevention	4	0.71	77	8.19
Carcinogenesis Mechanisms	0	0	90	10.01
Diet and Nutrition	0	0	50	4.83
Molecular Carcinogenesis	0	0	256	32.56
Smoking and Health	2	0.30	12	1.74
Research Resources	9	1.28	0	0
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TOTAL	15	2.29	485	57.33

SUMMARY REPORT

BIOLOGICAL AND CHEMICAL PREVENTION

The Biological and Chemical Prevention component of the Branch is responsible for research on agents that can inhibit, arrest, reverse, or delay the development of cancer in humans. Agents can derive from naturally occurring products such as foods consumed by man, from chemical synthesis, or from various biological sources. At the present time there are 73 grants in this program area with FY86 funding of approximately \$8.19 million and 4 contracts with FY86 funding of approximately \$0.71 million.

For the purpose of this report the research grants have been classified into Natural Inhibitors and Micronutrients (22), Antioxidants (8), Retinoids (20) and Miscellaneous (6) areas. They support diverse types of studies including the experimental inhibition of carcinogenesis, the inhibition or suppression of malignant transformation in culture, mechanisms of action and metabolism of preventive agents, synthesis of chemopreventive compounds, structure-function relationships, and pharmacologic disposition. The most frequently used experimental approach is to study inhibition of carcinogenesis induced by chemical, physical and biological agents, against several stages of the tumorigenic process, and against the development of cancer at many organ sites. The modifying effects of anticarcinogens are investigated relative to a large number of biochemical and biological endpoints, which, in addition to tumorigenesis and transformation themselves, include the activity of the mixed function oxidase system, free radical generation and quenching, cell proliferation, differentiation, activation/detoxification of carcinogens, DNA damage and repair, binding proteins or receptors for preventive agents, preneoplastic states and selective attack and prevention of oncogene-specific neoplastic disease.

A new program initiative this year was an RFA for Cooperative Agreements entitled "NATIONAL COLLABORATIVE CHEMOPREVENTION PROJECTS." The purpose of the resulting awards is to provide a mechanism to enhance and expand multidisciplinary/interdisciplinary investigations in chemoprevention through a funding mechanism that would permit combination of diverse research expertise from one or more institutions and the facilitating resources of the NCI. The PROJECTS are envisioned to have the capacity to generate new approaches and strategies in the inhibition or suppression of the carcinogenic process and to bridge development from individual grant/contract-supported work up to the stage of preclinical/clinical testing of new agents for the protection or prevention of neoplasia. Three National Collaborative Chemoprevention Project awards will be made in FY86. These projects will investigate the role of dehydroisoandrosterone in chemoprevention, the efficacy and biochemistry of vitamin D and vitamin D analogs in preleukemia and leukemia, and the design, synthesis and investigation of nucleophilic, nontoxic trapping agents for prevention of gastric and colon cancer.

Contracts supported by this component deal with studies on the chemoprevention of bladder cancer, the toxicity and pharmacology of preventive agents and inhibition, ameliorization and mechanisms of agent toxicity. Research accomplishments on a number of these endeavors are detailed later in this report.

Grants Activity Summary

Natural Inhibitors of Carcinogenesis: It is well known that a variety of fruits, vegetables, seeds, nuts and other edible plant materials consumed by man contain antimutagenic and anticarcinogenic substances. Detection of these substances has resulted from experiments employing the foods themselves, extracts from the foods, and in some cases chemically-defined classes of compounds demonstrated by natural product isolation and identification to be present in various edible plant categories. Increasing interest and attention are being devoted to the detection, isolation, purification and chemical identification of these substances, and to the range of activities which they possess in inhibiting the carcinogenic process. These latter studies seek to establish not only the species, organs and tissues in which inhibition of carcinogenesis can be demonstrated, but also the stages in the carcinogenic process which can be inhibited by these naturally-occurring substances. Some substances, for example, are known to act as both blocking agents and suppressing agents. Blocking agents have been defined as compounds that inhibit carcinogenesis by preventing carcinogenic compounds from reaching or reacting with critical target sites in the tissues. Suppressing agents have been defined as inhibitors that act subsequent to exposures to carcinogenic agents that otherwise will produce cancer. Many of these studies include efforts directed at determination of mechanisms of anticarcinogenesis.

One of the exciting areas involving natural inhibitors of carcinogenesis, and one in which very little previous work has been done, is that of citrus fruit oils. It has been reported previously that orange oil has a potent inducing effect upon the glutathione(GSH) S-transferase activities in both the liver and the small intestine of the ICR/Ha mouse. Furthermore, dietary orange oil inhibits neoplasia at several organ sites and under several different conditions. As a blocking agent, dietary orange oil inhibits benzo(a)pyrene (B(a)P)-induced neoplasia of both the forestomach and lung of female ICR/Ha mice. In fact, under these conditions where modulation of host susceptibility to subsequent carcinogenic exposure is obtained, orange oil produces the most potent inhibitory effect against B(a)P of any of the crude natural products studied to date. Most interesting, as a suppressing agent, orange oil suppresses 7,12-dimethylbenzanthracene-induced tumor formation in the female Sprague-Dawley rat. For both the blocking action in mice against BaP-induced gastric neoplasms and the suppressing action in rats against DMBA-induced mammary tumors, the percent of animals with tumors as well as the number of tumors per animal were decreased. It is further noteworthy that dietary dehydrated citrus pulp has been shown to inhibit azoxymethane-induced tumorigenesis in both colon and small intestine of Fischer F344 rats, as well as tumorigenesis induced in small intestine by 3,2'-dimethyl-4-aminobiphenyl. Finally, whole oranges provided in the diet of ICR/Ha mice also appear to protect in a blocking experiment against the induction of B(a)P-induced neoplasia of the forestomach and lungs. However, the observed protection in these cases was not statistically significant. On the other hand, whole oranges fed prior to and simultaneously with exposures to 1,2-dimethylhydrazine in the female CF₁ mouse have most remarkably inhibited neoplasia of the large bowel. In this case, the percent of mice with colonic tumors as well as the number of tumors per mouse were decreased by about 90%. In the orange-fed group of 17 mice, only one mouse had a single tumor, while 17 mice in the control group of 31 had 27 tumors.

A series of recent grant-supported studies has extended and added entirely new knowledge on the chemopreventive capacity of orange oil and its constituents against mammary carcinogenesis. One of these studies has employed the alicyclic monoterpene, d-limonene (C₁₀ H₁₆), the major component of citrus peel oils, and a

widely distributed product of the plant mevalonate pathway. In this study of mammary anticarcinogenesis in the Huggins model, d-limonene was fed continuously in the diet at two levels for 28 weeks beginning one week prior to a single dose of 7,12-dimethylbenz(a)anthracene (DMBA).

D-limonene feeding continued until sacrifice 27 weeks post-carcinogen administration. It should be noted that d-limonene is known to be completely metabolized within 48 hours of feeding, with most of its metabolites eliminated via urinary excretion. Ten metabolites have been previously characterized in rat urine. A significant reduction in carcinogenesis was observed at both 0.1% and 1% dietary d-limonene, an effect due mainly to an increase in latency. For example, a maximum 72% reduction in mammary tumor incidence was seen at 18 weeks post-DMBA administration. Furthermore, this major orange oil constituent caused regression of frank mammary tumors. No toxicity was observed in these rats even at the 1% level in the diet, with the rate of weight gain being identical in treated and control groups as well as hematocrit, total white cell, and differential counts being normal. It is interesting to note that a report in the literature 10 years ago indicated the absence of acute toxicity in humans given 20 grams of d-limonene in a single feeding.

These results with the cyclic monoterpene d-limonene have been extended by this same group of investigators. As reported above, this compound inhibits the development of mammary cancer when fed in the diet before, during and following administration of the rat mammary carcinogen DMBA, as well as causing the regression of frank mammary tumors. Yet, an early report published in 1959 had found d-limonene to be ineffective when tested for antitumor activity against three transplantable mouse tumor models (sarcoma 180, mammary adenocarcinoma CA755, and leukemia 1210). For these reasons, the therapeutic effectiveness of this monoterpene was investigated against primary, differentiated in situ tumors that had not been selected for transplantation. Again, a rat/DMBA mammary carcinogenesis model was selected. In this case, female (W/Fu x F344) F₂ rats were employed, again receiving a single dose of DMBA intragastrically at 50 days of age. Upon development of palpable mammary tumors and at an early stage of tumor development, rats were assigned and followed thereafter in pairs to a control diet and to a 10% d-limonene-supplemented diet. The therapeutic effectiveness of dietary d-limonene was then evaluated by following the growth of the first tumors in each pair. In addition, the development and growth of subsequently-appearing tumors were also followed. Results of this experiment indicate that d-limonene causes the regression of these chemically-induced, primary, differentiated, in situ mammary carcinomas. Furthermore, d-limonene also inhibited the formation of second or subsequent de novo breast tumors: control rats had an average of 3.4, while d-limonene-consuming rats had an average of 1.3 subsequent tumors (22).

This group of investigators has recently completed three additional significant studies on the anticarcinogenic and tumor-promoting properties of d-limonene, orange peel oil and other specific orange oil monoterpenes. Since these efforts have been published only in abstract/poster session form, they will not be discussed in detail at this time. However, results from these studies indicate that d-limonene is effective as both an anti-initiator and an "anti-progressor" in carcinogen-induced mammary tumorigenesis, that topically-applied orange peel oil is a weak mouse skin tumor promoter following DMBA initiation, but is not such a promoter when given in the diet, that d-limonene appears to have little or no promoting ability, and that neither orange oil nor d-limonene act as complete carcinogens in the mouse skin system.

Glutathione (GSH) S-transferase is a major detoxification system that protects the host from a wide variety of noxious xenobiotic compounds. This enzyme system catalyzes the binding of electrophiles to GSH. Since the reactive species of many chemical carcinogens are electrophiles, GSH S-transferase can play a significant role in carcinogen detoxification as well as in detoxifying other potentially harmful foreign compounds. The ability of many compounds to induce enhanced activity of this detoxifying system has been correlated with their anticarcinogenic capacity, and for the rodent mycotoxin carcinogen aflatoxin B₁, an excellent correlation has been observed between the degree of inhibition of DNA binding and the induction of hepatic GSH S-transferase activity by four antioxidants of three differing chemical classes. The use of such inducing ability has been most productive in detecting the presence of inhibitors of carcinogenesis in natural products and in identifying their chemical nature. An example of this approach has been the detection and identification of natural inhibitors of carcinogenesis in coffee beans. It has been found that consumption of diets containing powdered green coffee beans markedly enhances the GSH S-transferase activity in the liver and mucosa of the small intestine in the mouse. Defined types of green coffee beans from Brazil, Columbia, Guatemala, Mexico and Peru all have comparable enzyme-inducing ability. Roasting coffee beans does not result in a loss of the enhancing effect upon this detoxifying system. Commercial blends of roasted coffee and instant coffee have approximately 50% of the enhancing activity of the defined coffee beans from the specific countries studied, and decaffeinated instant coffee shows an enhancing effect similar to that of instant coffee. Furthermore, green coffee beans have the capacity to inhibit DMBA-induced mammary carcinogenesis in the rat both as a blocking "agent" when fed prior to carcinogen exposure and as a suppressive "agent" when fed during the post-initiation period. Two defined chemical compounds have been isolated and identified from coffee beans possessing significant GSH S-transferase enhancing activity. These compounds are chemically very similar to one another, differing by the presence or absence of a single double bond. They are cafestol palmitate and kahweol palmitate. These compounds are coffee oil constituents and are also terpenoid compounds, as discussed above for the orange peel oil compounds. In this case, the two compounds are diterpene esters. Diterpenes constitute a large, varied and widely distributed family of compounds occurring in plants. Recently, structure-activity studies have been performed with cafestol and kahweol directed at elucidating what molecular structural features of these diterpenes are involved in enzyme-enhancing activity and protective effects. Studies on structure-activity relationships are important in determining mechanism(s) of action and for guiding synthesis of additional compounds with optimal inhibitory properties. Structure-activity data may also be of value in identifying other naturally-occurring diterpenes likely to have protective properties.

Of the two diterpenes, kahweol is the better inducer of GSH S-transferase. This compound has an additional double bond on the A-ring of the diterpene nucleus. Two functional moieties exist on the diterpene nucleus of these compounds: a glycol function (at C16 and C17) and a furan ring (attached to the A-ring at C3 and C4). Chemical modifications were carried out on the glycol group of these two diterpenes to give the ketones, the alcohols, the acetates, the hydrocarbons and a 16-hydroxy, 16-methyl derivative. The derivatives were then tested for their ability to enhance the GSH S-transferase activity in the liver and small bowel mucosa of ICR/Ha mice. Results indicate that all these chemical alterations of the glycol group decrease, but do not eliminate, the enhancing effects of both these diterpenes on this detoxifying enzyme activity. In addition, kahweol and cafestol increase GSH levels. The chemically-modified derivatives in most cases were also less potent in this regard than the parent compounds.

Additional chemical modification of cafestol at the furan moiety has demonstrated that the major determinant of inducing activity for increased GSH S-transferase activity resides in the furan moiety. Catalytic hydrogenation of cafestol to either the dihydro compound (wherein the exposed double bond of the furan moiety is saturated) or to the tetrahydro compound (wherein both the double bonds of the furan moiety are saturated) results in loss of enhancing capacity of this diterpene (and its acetate ester) for GSH S-transferase activity in both the liver and small bowel mucosa of ICR/Ha mice. An unexpected finding, in addition to the valuable information on the need of the furan structure for inducing activity, is that the inducing characteristics of cafestol for increasing the levels of GSH were retained by these saturated dihydro and tetrahydro derivatives. These results suggest that chemical protection of the furan moiety from metabolic attack may be a productive strategy for inducing more potent inducers of enhanced GSH S-transferase activity and more effective protection from chemically-induced carcinogenesis (71).

Three very interesting inhibition studies have been performed on the capacity of cafestol palmitate to protect against chemical carcinogenesis. Two of the experiments presumably involve modification of host status to subsequent carcinogen exposure. In these experiments, cafestol palmitate was acting as a blocking or protective agent. In the first experiment, the diterpene ester was given once a day orally for three days to A/J mice, which then received an oral dose of B(a)P on the fourth day. This procedure resulted in a one-third reduction in the number of induced pulmonary adenomas. In the second protection experiment, the mice were given a single administration of cafestol palmitate 4 hours prior to BaP. This procedure resulted in a 50 percent reduction in the number of pulmonary adenomas. If short time intervals on the order of minutes should prove effective, then these results are fascinating in that they suggest that consumption of the diterpene ester shortly before the intake of a carcinogen with a meal (for example) could have a major impact in protecting against the development of cancer.

In the third experiment, cafestol palmitate was tested for its capacity to inhibit mammary carcinogenesis in the Huggins model when given in the post-initiation period. Under these conditions, cafestol palmitate did not suppress mammary tumor formation. This experiment has particular interest when it is recalled that in this DMBA-induced rat mammary carcinogenesis model, previous results showed that administration of coffee beans themselves, or a petroleum ether extract of the beans, subsequent to DMBA did suppress mammary tumor formation. These results in aggregate emphasize, again, the usefulness of the GSH S-transferase biochemical assay for detection of blocking agents in complex natural products, extracts or purified chemical compounds. They also demonstrate the need for a similar reliable short-term assay(s) for detection of suppressive agents effective in post-initiation phases of carcinogenesis (71). Such an assay has recently been suggested by this same group of investigators.

Another interesting area of natural inhibitors of carcinogenesis is that of onion and garlic oils. Recently, a report appeared describing the ability of both onion and garlic oils to inhibit mouse skin papilloma formation in two-stage carcinogenesis employing DMBA as the initiator and phorbol myristate acetate (PMA) as the promoter. Moreover, results in cell culture have indicated that onion oil inhibits PMA-enhanced transformation of benzo(a)pyrene-treated NIH3T3 cells. However, both of these oils appear not only to enhance PMA-induced hyperplasia in mouse skin, but also to stimulate the growth of 3T3 cells. Also at higher doses in mouse skin, onion oil by itself produces a small degree of hyperplasia. These

potentially adverse indications have prompted additional investigations on the biological actions of these oils. Results of these recent studies indicate that (1) garlic oil does not promote DMBA-initiated tumorigenesis in ICR/Ha mouse skin when given topically 3x/week for 50 weeks in 1 mg doses; (2) onion oil appears to be a weak topical promoter for DMBA-initiated mouse skin under the same circumstances producing less than one papilloma per mouse at 330 days and a single carcinoma at 297 days; (3) with large 10 mg doses, however, onion oil produced a cumulative total of 63 tumors and 7 carcinomas at 345 days, equivalent to 3.2 and 0.3/mouse, respectively; and (4) the onion oil inhibition of PMA-promoted skin papilloma formation, and in fact, inhibition of carcinoma formation resulting from papilloma progression, is critically dependent upon the interval between onion oil and PMA topical applications. For carcinoma formation, inhibition seems only to occur when onion oil is applied 0.5 hour after PMA application. When applied 0.5, 1.0 or 2.0 hours before, or at 1.0 or 2.0 hours after PMA, enhancement of carcinoma formation actually occurs. One simple explanation of these results is that onion oil is a complex mixture, and the inhibitory and stimulatory effects reside in separate molecules. However, alternate explanations are possible, considering this and other data not reported here, including the hypothesis that onion oil may be a weak carcinogen (4).

Additional studies with these oils and with some of the known chemical constituents of these oils have been directed at their mechanisms of action in producing these varied biological effects, particularly the inhibition of skin tumor promotion by both onion and garlic oils. A current hypothesis indicates that oxidative mechanisms play a determining role in the process of tumor promotion. This hypothesis derives from (1) the fact that free oxygen radical-generating compounds exhibit tumor-promoting activities and various tumor promoters stimulate the production of reactive oxygen species, (2) the fact that diverse antioxidants and free radical scavengers inhibit the oxidative challenge and some of the early molecular events linked to tumor promotion, and (3) that agents which stimulate cellular detoxifying enzyme systems for activated oxygen species (such as superoxide dismutase, catalase and GSH peroxidase) also can protect from adverse oxidative consequences and inhibit known biochemical events associated with tumor promotion. Recent data in this regard indicate that both onion and garlic oils are potent inhibitors of soybean lipoxygenase. Lipoxygenase is a key enzyme in the metabolism of arachidonic acid to activated lipid hydroperoxides and is an enzyme known to be stimulated by the potent skin tumor promoter, PMA. Moreover, a minor component of onion oil, dipropenyl sulfide, has been identified as an active, reversible, as well as irreversible, inhibitor of soybean lipoxygenase. On the other hand, the major constituent of onion oil, dipropyl disulfide, has been found neither to inhibit this enzyme nor to inhibit tumor promotion. These biochemical studies have employed linoleic acid (C18:2, omega-6) as substrate for the enzyme. It is interesting to note that the onion oil active inhibitor dipropenyl sulfide (C-C=C-S-C=C-C) is an analogue to the cis, cis-1,4-pentadiene (-C=C-C-C=C-) moiety of the linoleic acid substrate (4).

A further set of studies has been directed at elucidating the mechanisms of action in inhibition of tumorigenesis by these oils. These studies have employed isolated mouse epidermal cells in suspension culture. These suspensions consist predominately of small, basal epidermal cells which are morphologically well preserved. Fibroblast contamination was about 5%. Garlic oil, onion oil and the onion oil-active inhibitor of lipoxygenase, dipropenyl sulfide, when added to these cultures, all increase GSH peroxidase activity, either in the presence or the absence of the potent skin promoter PMA. The stimulatory effects on epidermal GSH peroxidase activity are concentration-dependent and long-lasting, thus

abolishing the prolonged inhibition of this enzyme produced by the tumor promoter. Garlic oil, onion oil and dipropenyl sulfide also inhibit the increase in ornithine decarboxylase produced by this phorbol ester, as well as the nonphorbol ester tumor promoters mezerein, benzoyl peroxide, anthralin and hydrogen peroxide. These oils and dipropenyl sulfide are also effective at increasing GSH peroxidase activity in the presence of these latter promoters. Finally, garlic oil, onion oil and dipropenyl sulfide treatments of these epidermal cells significantly inhibit the sharp decrease in the ratio of intracellular reduced (GSH)/oxidized (GSSG) glutathione produced by PMA. These results suggest that enhancement of the GSH-dependent antioxidant protective system may be an additional mechanism whereby garlic and onion oils inhibit skin tumor promotion (4).

Protease inhibitors constitute another fascinating area of natural inhibitors of carcinogenesis. It has been known that several different protease inhibitors have the ability to suppress the malignant transformation of cells in vitro, such as C3H/10T 1/2, A31-11 mouse BALB/3T3 and human diploid (AG-1522) cells. Inhibitors of chymotrypsin are among the most potent protease inhibitors known to inhibit malignant transformation. One such potent chymotrypsin inhibitor is the Bowman-Birk inhibitor (BBI) derived from soybeans. It is known that this protease inhibitor effectively suppresses malignant transformation induced in vitro by radiation and the chemical carcinogens, benzo(a)pyrene and beta-propiolactone (both with and without the cocarcinogen, pyrene). This suppression of transformation has been shown in several different cell systems and has an irreversible effect on the transformation process, even when present only for a short period of time. It is effective in suppressing radiation-induced transformation even when added to cultures many days after exposure. BBI is effective in its ability to suppress transformation when present at a concentration as low as 1 ng/ml. It is only the chymotrypsin inhibitory region (and not the trypsin inhibitory region) of the Bowman-Birk molecule which appears to be involved in suppression of transformation in vitro. The BBI, when ingested in the diet, is known to reach the colon in an active form. And recently, it has been demonstrated that dietary addition of an extract of soybeans containing the Bowman-Birk protease inhibitor protects against dimethylhydrazine (DMH)-induced adenomatous tumors of the colon in male CD₁ mice. This animal model for colon carcinogenesis induces adenocarcinomas of the colon and rectum in rodents and is considered to resemble human colorectal cancer. The BBI extract employed in these studies is known to contain four other separate protease inhibitors in addition to BBI, as well as soybean trypsin inhibitor. These other protease inhibitors are similar to BBI in molecular weight and trypsin inhibitory activity. The extract was provided at a level of 0.5% in a purified diet containing 24.5% casein as the protein source. Two control groups received DMH: one on a diet containing 25% casein and the second a diet containing 15% casein (with an additional 10% carbohydrate replacing the protein). No colon tumors appeared whatsoever in the DMH-treated animals fed the protease-inhibitor-containing diet. Animal weights were comparable in all three DMH-treated groups, throughout the experiment as well as at necropsy. Furthermore, food intake was comparable in all groups. It is possible that a decrease in protein utilization could have occurred in the experimental group of mice receiving the protease inhibitor extract. The DMH-treated group receiving 15% protein was a control on this possibility and may be most comparable to the protease inhibitor-ingesting group. Fifteen percent protein intake is near the minimum recommended for a standard mouse diet, while the 25% protein diet was designed to contain more than the standard amount of protein so as to compensate for any decrease in protein utilization caused by consumption of the protease inhibitor diet. However, tumor incidence (14% and 17%, respectively) in these control, non-BBI-fed groups was approximately the same whether they consumed a 5%

or a 25% casein diet. Combining the tumor incidence in these two groups, 15 percent (7/46) of the mice had tumors (a total of 11) in the control vs. zero percent in the BBI extract group (p less than 0.05) (28).

Not only was a lack of toxicity indicated for this BBI preparation by body weight data, but also by the lack of change in the pancreas/body weight ratio and the occurrence of normal pancreas histology on pancreata from all animals in the experiment. These mice had been fed the BBI extract for 32 weeks. In the rat, high levels of dietary soybean trypsin inhibitor for long periods of time are known to lead to pancreatic cell hypertrophy and hyperplasia, and in a few rats, pancreatic cancer. However, these pancreatic changes in the rat are thought to be caused specifically by trypsin inhibition and not by chymotrypsin inhibition. Pancreatic changes such as these are not expected to develop in man nor in mice, since the human cationic trypsin is not strongly inhibited by soybean trypsin inhibitors. Furthermore, in additional experiments, an unchanged pancreas/body weight ratio and normal pancreas histology were again found for mice consuming 0.5% BBI extract for 1.5 years, a period of time encompassing most of their normal life span (28). This apparent lack of toxicity in the mouse, even with chronic ingestion of this protease inhibitor extract under conditions known to inhibit DMH-induced colon carcinogenesis, is a compelling aspect in consideration of programmatic development of this class of anticarcinogenic agent.

Another interesting area in natural product inhibition of carcinogenesis is that of the cruciferous vegetables and specific chemical constituents contained in these vegetables known to inhibit tumorigenesis. The complexities in anticarcinogenesis experiments and interpretations from them are well illustrated by recent results obtained with the chemical compound indole-3-carbinol (I3C), a natural constituent of cruciferous vegetables such as broccoli, Brussels sprouts, cauliflower and cabbage. It is known that I3C is an inhibitor, in animal model systems, of carcinogenesis induced by polynuclear aromatic hydrocarbons or aflatoxin B₁ (AFB₁) when administered prior to or during carcinogen exposure. Recent studies have now shown that, in the rainbow trout model system for aflatoxin-induced hepatocarcinogenesis, preinitiation exposure to I3C reduces AFB₁-initiated hepatocellular carcinoma incidence as previously reported, but that post-initiation administration of I3C strongly enhances tumor incidence above that produced by AFB₁ alone (2). Further, previous experiments in the trout model have indicated that continuous feeding of I3C before, during and 6 weeks after AFB₁ feeding reduced tumor incidence at approximately one year post-AFB₁ administration. On the other hand, a preliminary report has indicated that such continuous feeding of I3C before, during and after DMH administration to F344 rats enhances induced colon carcinogenesis. Further, in SENCAR mouse epidermal preparations, I3C appears to further enhance the TPA-enhancement of ornithine decarboxylase activity, but does not itself induce an increased activity of this enzyme associated with the promotion phase of carcinogenesis.

Mechanisms of I3C inhibition of AFB₁ trout hepatocarcinogenesis have been investigated via possible effects on carcinogen metabolism, distribution, and binding to DNA. Prefeeding I3C decreased the binding of radiolabeled AFB₁ to trout liver DNA by 70% throughout the 3 week period investigated immediately following carcinogen administration. A 24 hour distribution study of injected labeled AFB₁ to fish prefed I3C showed less radioactivity in the blood and liver at all times examined. These reductions in the I3C-treated fish were due primarily to reduced levels of AFB₁ bound to red blood cell DNA, reduced levels of the primary metabolite, aflatoxicol, in the plasma and decreased levels of AFB₁ and its polar metabolites in the liver. In contrast, total radioactivity was

significantly elevated in the bile of I3C-fed fish, a result of a sevenfold increase in aflatoxin-M₁ glucuronide in the treated fish. Bile levels of AFL glucuronide, the primary conjugate present in control fish, were no different in the I3C-fed fish. Similarly, no differences were seen in the radioactivity remaining in the carcasses of control and treated fish. Though a significant weight loss was seen in the I3C-fed fish used for the distribution studies, these results as a whole indicate that I3C inhibition of AFB₁ hepatocarcinogenesis in the trout may involve substantial changes in the pharmacokinetics of carcinogen distribution, metabolism and elimination, leading to a significantly reduced level of initial hepatic nuclear DNA damage in vivo (2).

A new chemopreventive class of chemical compounds has recently emerged possessing great potential for inhibition of carcinogenesis. These compounds are substituted 1,2-dithiol-3-thiones. Dithiothiones are known to be present in cruciferous vegetables. Consumption of these vegetables is well known to be associated with a reduced cancer incidence in man and, in the experimental setting, with an inhibition of carcinogenesis in animal tumorigenesis models. Some dithiothiones are used as antischistosomal agents, cholagogues and antioxidants in rubber, oils, greases and metals. Substituted 1,2-dithiol-3-thiones, such as the synthetic compound oltipraz (5-[2-pyridinyl]-4-methyl-1,2-dithiol-3-thione) have been shown to protect against the acute and chronic toxicities of several xenobiotic compounds in rodents. Oltipraz is known to be nonmutagenic and appears to be very nontoxic. Pretreatment with this substituted dithiothione has recently been found to confer several chemoprotective benefits to carcinogen-treated rodents: (1) pretreatment with oltipraz has been shown to reduce, considerably, benzo(a)-pyrene-induced neoplasia of mouse lung (80%) and forestomach (60%); (2) dietary administration (0.1%) of oltipraz for 2 weeks to male F344 rats induced significant hepatic increases in reduced GSH, glucose-6-phosphate-dehydrogenase, glutathione reductase, glutathione S-transferase, UDPG-transferase, epoxide hydrolase and cytochrome P-450. These enzymes are known to be involved in AFB₁ activation and detoxification (see below); (3) such 2 week dietary administration is also known to significantly reduce the accumulation of aflatoxin B₁ in liver and kidney measured 2 hours after dosing intraperitoneally. DNA binding of this mycotoxin carcinogen was also reduced. In this latter case, oltipraz reduced overall binding of AFB₁ to liver DNA by 76% and to kidney DNA by 64% measured 2 hours after AFB₁ administration. In both of these tissues, covalent adducts in the oltipraz-treated rats were still principally 8,9-dihydro-8-(N⁷-guanyl)-9-hydroxyaflatoxin B₁ and related adducts just as in control animals; (4) dietary (0.1%) oltipraz fed to male F344 rats one week before, 2 weeks during, and one week after aflatoxin B₁ treatment inhibits the formation of gamma glutamyl transpeptidase (GGT)-positive foci determined after 4 months. Both number and volume of foci were reduced by approximately 95%. Since in other experiments, rats fed oltipraz showed a rapid induction in GSH S-transferase specific activity, elevated GSH S-transferase mRNA levels, increased biliary elimination of AFB₁-glutathione conjugate, and reduction in hepatic DNA adduct formation, the inhibiting effect of oltipraz on GGT-positive foci production was hypothesized to be related to induction of detoxication enzymes; and (5) the chemoprotective effect of oltipraz on AFB₁-induced GGT-positive foci was demonstrated (in the same protocol as above) down to dietary levels of 0.01%. In the range 0.01 to 0.1%, the number and percent volume of GGT foci were reduced from 70 to 98%. An interesting finding in this dose-response study is that although 0.1% of the substituted dithiothione rapidly induces both phase I and phase II enzymes (particularly the glucuronyl and glutathione S-transferases), the low 0.01% dose selectively induces only phase I enzyme activities. Phase I reactions, according to Williams, introduce polar groups into xenobiotic compounds and are mostly

oxidative reactions, though a few are reductive. Additional experimentation will be required to determine the relative importance of altered activation and detoxification pathways in dithiothione modulation of hepatic aflatoxin B₁-induced GGT-positive liver foci and hepatocarcinogenesis (29).

Contracts Activity Summary

Few investigations have studied possible adverse effects resulting from prolonged administration of chemopreventive agents. A recent discovery of potentially serious toxicity has occurred during the course of a study on the chemopreventive efficacy of the retinoid, 13-cis-N-ethylretinamide. The investigation was designed to determine the anticarcinogenic activity of this retinoid in the post-initiation period of butylhydroxybutyl-nitrosamine-initiated bladder cancer in the B6D2F1 hybrid mouse. Three levels of carcinogen were administered to separate groups of mice which were subsequently given or not given dietary retinoid. Groups of mice not receiving carcinogen, but administered dietary retinoid, were also maintained. A most unexpected finding in these investigations has been the observation of hepatocellular carcinomas, adenomas and other liver lesions in very high incidence in this mouse model by prolonged dietary administration of this retinoid. This induction of liver cancer results from feeding the retinoid only; it does not appear to be tumor promotion following the nitrosamine initiation. In addition, "spontaneous" liver lesion frequency in noncarcinogen, nonretinoid, placebo-fed animals appears to be very low (74). Prolonged administration of 13-cis-NER (68 to 82 weeks) also appears to be necessary for induction of these liver lesions, since previous studies in this mouse model with this particular retinoid, fed for only 22 weeks, did not result in grossly-observable abnormal lesions. Studies in experimental anticarcinogenesis and considerations of possible continuous human exposure to cancer-causing or -enhancing agents indicate that such prolonged or even permanent administration of chemopreventive agents might be necessary, at least during some periods of the human lifetime or stages of the carcinogenic process. Such considerations coupled with the unexpected result above (and others in the recent literature) emphasize the critical need for development of nontoxic chemopreventive/chemoprotective agents, long-term experimental studies on agent toxicity, development of early indicators of later-appearing serious toxicities and their prevention or ameliorization, and investigations into the mechanisms of agent-induced toxicities. These types of investigations are currently underway (75, 76).

BIOLOGICAL AND CHEMOPREVENTION

GRANTS ACTIVE DURING FY86

<u>Investigator/Institution/Grant Number</u>	<u>Title</u>
1. AWASTHI, Yogesh C. Univ. of Texas Med. Br. (Galveston) 2 R01 CA27967-07	Mechanism of Anticarcinogenic Effect of Antioxidants
2. BAILEY, George S. Oregon State University 5 R01 CA34732-03	Mechanisms of Inhibition of Chemical Carcinogenesis
3. BANERJEE, Mihir R. University of Nebraska (Lincoln) 5 R01 CA25304-06	Chemical Carcinogenesis Mammary Gland Organ Culture
4. BELMAN, Sidney New York University 5 R01 CA38156-02	Tumor Control by Onion, Garlic, and a Protease Inhibitor
5. BENEDICT, William F. Children's Hospital of Los Angeles 5 R01 CA31574-04	Ascorbic Acid Transformation and Oncogenic Progression
6. BENSON, Ann M. Univ. of Arkansas Med. Sci. (Little Rock) 5 R01 CA38791-02	Modulation of Enzyme Profiles by Anticarcinogenic Agents
7. BERNSTEIN, Isadore A. University of Michigan at Ann Arbor 5 R01 CA32470-03	Mechanism for Retinoid Neutral- ization of Tumor Promotion
8. BERTRAM, John S. University of Hawaii at Manoa 5 R01 CA39947-02	Inhibition of In Vitro Trans- formation by Retinoids
9. BRESNICK, Edward University of Nebraska Medical Center 5 R01 CA38150-02	Cruciferae and Carcinogenesis
10. BRINCKERHOFF, Constance E. Dartmouth College 5 R01 CA32476-03	Action of Retinoids on Synovial Cells
11. BROWN, Neal C. University of Massachusetts Med. Sch. 1 R01 CA40893-01	Novel Inhibitor-Probes of the <u>ras</u> Oncogene Protein P21
12. CASSADY, John M. Purdue University West Lafayette 5 R01 CA38151-02	Novel Natural Inhibitors of Carcinogenesis

13. CHOPRA, Dharam P
Southern Research Institute
1 R01 CA35593-01A2
Mechanism of Retinoid Action
Against Prostate Lesions
14. CHUNG, Fung-Lung
American Health Foundation
2 R23 CA32272-03
Screening for Inhibitors of
N-Nitrosamine Carcinogenesis
15. COPE, Frederick O.
Southern Research Institute
1 R01 CA40894-01
Retinoid Receptor Control in
Cytodifferentiation
16. CURLEY, Robert W.
Ohio State University
1 R01 CA40967-01
Affinity Probes for the Retinoic
Acid-Binding Proteins
17. CURPHEY, Thomas J.
Dartmouth College
5 R01 CA 32478-03
Pancreatic Cancer and Retinoids-
Model and Mechanism
18. DAWSON, Marcia I.
SRI International
5 R01 CA32428-05
Retinoid Tumor Inhibitory
Activity-Toxicity Probe
19. DAWSON, Marcia I.
SRI International
5 R01 CA30512-05
Novel Retinoids for Chemo-
prevention of Cancer
20. DOERING, William V.
Harvard University
1 R01 CA41325-01
Semi-Rigid Conjugated Polyenes
as Model Anticarcinogens
21. DURHAM, John P.
West Virginia University
5 R01 CA37060-02
Calcium/Lipid Protein Kinase in
Myeloid Differentiation
22. GOULD, Michael N.
University of Wisconsin (Madison)
5 R01 CA38128-02
Anticarcinogenic Agents in
Orange Peel Oil
23. GRUBBS, Clinton J.
University of Alabama at Birmingham
7 R01 CA41974-01
Chemoprevention of Cancer Caused
by Anticancer Agents
24. HADDOX, Mari K.
Univ. of Texas Hlth. Sci. Ctr. (Houston)
5 R01 CA 32444-03
Mechanism of Retinoid Inhibition
of Cell Proliferation
25. HILL, Donald L.
Southern Research Institute
5 P01 CA34968-02
Development of Chemopreventive
Retinoids
26. HULTIN, Theresa A.
IIT Research Institute
1 R23 CA40521-01
Pharmacogenetics of Retinoid
Inhibition of Liver Cancer

27. JOHNSON, Eric F.
Scripps Clinic and Research Foundation
5 R01 CA 34910-03
Modulation of Carcinogen
Activation/Detoxification
28. KENNEDY, Ann R.
Harvard University
5 R01 CA38246-02
Suppression of DMH-Induced Colon
Cancer
29. KENSLER, Thomas W.
Johns Hopkins University
1 R01 CA39416-01
Modulation of Aflatoxin Toxicity
by Dietary Antioxidants
30. KENSLER, Thomas W.
Johns Hopkins University
5 R01 CA36380-03
Biomimetic Superoxide Dismutases
as Antitumor Promoters
31. KOEFFLER, H. Phillip
University of California (Los Angeles)
2 R01 CA33936-04
Action of Retinoids on Myeloid
Leukemia Cells
32. KRUMDIECK, Carlos L.
University of Alabama at Birmingham
1 R01 CA40834-01
Folate Deficiency--Preventable
Risk of Cancer
33. LAM, Luke K. T.
University of Minnesota (Mnpls-St Paul)
5 R01 CA38932-02
Carcinogenesis of Butylated
Hydroxyanisole
34. LANDOLPH, Joseph R.
University of Southern California
1 R01 CA 41277-01
Oxygen Radicals, Prostaglandins,
and Chemical Transformation
35. LIEHR, Joachim G.
Univ. of Texas Med. Br. (Galveston)
1 R01 CA 43232-01
Prevention of Estrogen-Induced
Tumors by Chemical Means
36. LIPKIN, Martin
Sloan-Kettering Institute for Can. Res.
1 R01 CA40876-01
Natural Inhibitor of Colonic
Cell Damage
37. LONGNECKER, Daniel S.
Dartmouth College
5 R01 CA38131-02
Glycerol Monooleate and
Pancreatic Carcinogenesis
38. LOTLIKAR, Prabhakar D.
Temple University
1 R01 CA40885-01
Mechanism of Anticarcinogenesis
by Antioxidants
39. LUDLUM, David B.
Albany Medical College of Union Univ.
5 R01 CA 32446-03
Repair of Carcinogenic Lesions in
DNA
40. MC CORMICK, Anna M.
Univ. of Texas Hlth. Sci. Ctr. (Dallas)
2 R01 CA31676-04
Metabolism of Chemopreventive
Retinoids

41. MC CORMICK, David L.
IIT Research Institute
1 R01 CA40874-01
Arachidonic Acid Metabolism and
Cancer Chemoprevention
42. MEDINA, Daniel
Baylor College of Medicine
5 R01 CA11944-14
Biology of Mammary Preneoplasias
43. MEDINA, Daniel
Baylor College of Medicine
5 R01 CA 32473-03
Selenium Inhibition of Mouse
Mammary Tumorigenesis
44. MEHTA, Rajendra G.
IIT Research Institute
5 R01 CA 34664-03
Hormone and Retinoid Interaction
in Mammary Tissue
45. MOORE, Malcolm A.
Sloan-Kettering Institute for Can. Res.
5 R01 CA 32516-03
Mechanisms of Biological Prevention
of Leukemogenesis
46. NAPOLI, Joseph L.
State University of New York at Buffalo
7 R01 CA42092-01
Determinants of Vitamin A
Homeostasis
47. NILES, Richard M.
Boston University
5 R01 CA32543-04
Regulation of Growth and Differ-
entiation by Retinoids
48. ONG, David E.
Vanderbilt University
5 R01 CA20850-09
Cancer and Vitamin A
49. PRASAD, Kedar N.
University of Colorado Hlth. Scis. Ctr.
1 R01 CA40619-01
Mechanism of Action of Vitamin E
on Cancer
50. PROUGH, Russell A.
University of Louisville
5 R01 CA 43839-02
Inhibitor Effects on Monooxygenase
Function
51. REDDY, Chinthamani C.
Pennsylvania State University
1 R01 CA37979-01A2
Vitamin E, Selenium, and Fatty
Acid-Dependent B(a)P Oxidation
52. REDDY, Janardan K.
Northwestern University
2 R01 CA32504-04A1
Modification of Peroxisome
Proliferator Carcinogenesis
53. REINERS, John J.
Univ. of Texas System Cancer Center
1 R01 CA40823-01
Inhibition of Chemical Carcino-
genesis by Copper Chelates
54. REINERS, John J., Jr.
Univ. of Texas System Cancer Center
5 R01 CA34469-03
Inhibition of Chemical Carcino-
genesis by Interferon

55. ROGERS, Adrienne E.
Massachusetts Institute of Technology
5 R01 CA 32520-02 Anticarcinogenic Effects of
Selenium and Vitamin A
56. ROGERS, Adrienne E.
Boston University
7 R01 CA 39222-01 Azaserine Carcinogenesis Effects
of Methionine Choline
57. RUDDLE, Nancy H.
Yale University
5 R01 CA 32447-03 Lymphotoxin and Interferon
Inhibition of Carcinogenesis
58. SANI, Brahma P.
Southern Research Institute
1 R01 CA40756-01A1 Studies on Retinoic Acid-Binding
Protein
59. SCHWARTZ, Arthur G.
Temple University
1 R01 CA38574-01A1 Studies on Cancer Preventive
Effects of DHEA and Analogs
60. SHANKEL, Delbert M.
University of Kansas
1 R13 CA39971-01A1 International Conference -
Mechanisms of Antimutagenesis
61. SHERTZER, Howard G.
University of Cincinnati
5 R01 CA38277-02 Chemoprotection from N-Nitros-
amines by Dietary Indole
62. SLAGA, Thomas J.
Univ. of Texas System Cancer Center
5 R01 CA 34521-03 Inhibition of Tumor Promotion by
Antioxidants
63. THOMPSON, Henry J.
University of New Hampshire
5 R01 CA 32465-03 Breast Cancer Chemoprevention and
Polyamine Biosynthesis
64. THOMPSON, Henry J.
University of New Hampshire
5 R01 CA38265-02 Cancer Prevention and Vanadium
65. THOMPSON, John A.
University of Colorado at Boulder
1 R01 CA41248-01 Bioactivation of Dietary Phenols
by Hemoproteins
66. VERMA, Ajit K.
University of Wisconsin
1 R01 CA42585-01 Inhibit. of Ornithine Decarboxy-
lase Induction by Retinoid
67. VERMA, Ajit K.
University of Wisconsin (Madison)
5 R01 CA36323-02 Inhibition of Skin Tumor Promotion
68. WALL, Monroe E.
Research Triangle Institute
5 R01 CA38245-02 New Natural and Synthetic Inhibitors
of Carcinogenesis

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| 69. WANG, Alexander Y.
Univ. of Texas System Cancer Center
5 R01 CA35363-02 | Vitamin E and Cancer |
| 70. WANG, Sho-Ya
Dana-Farber Cancer Institute
1 R23 CA41274-01 | Teratocarcinoma Cells: Gene
Regulation by Retinoic Acid |
| 71. WATTENBERG, Lee W.
University of Minnesota (Mnpls-St Paul)
5 R01 CA37797-02 | Diterpenes as Inhibitors of
Carcinogenesis |
| 72. WEBB, Thomas E.
Ohio State University
5 R01 CA38125-02 | B-Glucuronidase Inhibition and
Chemical Carcinogenesis |
| 73. WOLF, George D.
Massachusetts Institute of Technology
5 R01 CA13792-09 | Vitamin A and Glycoproteins of
Skin Tumors |

CONTRACTS ACTIVE DURING FY 86

<u>Investigator/Institution/Contract Number</u>	<u>Title</u>
74. HICKS, R. Marian Middlesex Hospital Medical School N01-CP-05602	Chemoprevention of Epithelial Cancer by Retinoids (Bladder)
75. HILL, Donald L. Southern Research Institute N01-CP-41005	Studies on Toxicology and Pharmacology of Biological and Chemopreventive Agents
76. McCORMICK, David L. IIT Research Institute N01-CP-41063	Studies of Toxicology and Pharmacology of Biological and Chemopreventive Agents
77. MOON, Richard C. IIT Research Institute N01-CP-05718	Chemoprevention of Epithelial Cancer by Retinoids (Mammary Gland)

SUMMARY REPORT

CARCINOGENESIS MECHANISMS

The Carcinogenesis Mechanisms component of the Branch consists of studies relating to the metabolism, toxicity, physiological disposition, and mechanisms of action of carcinogens and their metabolites. Studies involving the synthesis of both known and suspect carcinogens or the development of derivatives for molecular structure-activity relationships are also included. Other studies deal with the hormone-related biochemistry of cancer and cancerous hosts, the identification of reactive metabolites and the isolation and characterization of carcinogen metabolizing enzymes. Currently, the research grant is the sole instrument of support for this area. In FY 1986 there was one program project grant (P01), one MERIT award (R37), one young investigator grant (R23), one conference grant (R13), and 85 traditional research grants (R01) with a total funding level of \$10.01 million.

Grants Activity Summary

Polycyclic Aromatic Hydrocarbons: Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants which may be responsible for some cancer induction in man. The biological properties of PAHs, such as mutagenicity, carcinogenicity, and covalent binding to cellular macromolecules, require metabolic activation to an electrophilic species. Metabolic activation of PAH may occur by two main pathways: monooxygenation and one-electron oxidation. Monooxygenation affords oxygenated metabolites by direct attack of an "oxene-like" activated oxygen generated by cytochrome P-450 monooxygenase. One electron oxidation produces radical cations. In an aromatic system a radical cation is formed by removal of one- π -electron, whereas one-n-electron oxidation of a phenol or amine, followed by rapid loss of a proton, yields a radical. Most PAH metabolites, such as epoxides, dihydrodiols, phenol-dihydrodiols, dihydrodiol-epoxides, triols and tetrols are optically active. The ultimate carcinogenic metabolite of benzo(a)pyrene (B(a)P), for instance, is a diastereomeric 7,8 dihydrodiol-9,10-epoxide of high optical purity. One project involves the elucidation of the stereochemical pathways of metabolism in the detoxification and activation of PAHs (90). Research efforts include the resolution of optical isomers and the elucidation of their absolute configurations. An HPLC method using a column packed with a chiral stationary phase (CSP), (R)-N-(3,5-dinitrobenzoyl)phenylglycine, ionically bonded to alpha-aminopropylsilanized silica was developed to resolve some dihydrodiol and tetrahydrodiol enantiomers of benz(a)anthracene (BA) and B(a)P directly. The direct resolution of 86 structurally related monomethyl, mono-ol, and the trans- and cis- diol enantiomers of unsubstituted and methyl-substituted BA and B(a)P was investigated using this CSP-HPLC procedure. The results indicated that structural factors, such as conformation, presence of a methyl substituent, molecular size and shape, and ring saturation all contribute to chiral interactions between the chiral stationary phase and the solutes.

This method has been applied to the determination of the optical purity and absolute configuration of the K-region epoxides formed in the metabolism of 1-methylbenz(a)anthracene, 7-methylbenz(a)anthracene, and 12-methylbenz(a)anthracene by rat liver microsomes. The K-region epoxides formed in the metabolism of BA by liver microsomes from untreated (control), phenobarbital (PB)-treated, and 3-methylcholanthrene (MC)-treated male Sprague-Dawley rats were also determined by CSP-HPLC to have a 5R,6S/5S,6R enantiomer ratio of 25:75, 21:79, and 4:96, respectively. The K-region 4,5-epoxide formed in the metabolism of B(a)P by the same

rat liver microsomal preparations contained a 4R,5S/4S,5R enantiomer ratio of 48:52 (control), 40:60 (PB), and 5:95 (MC), respectively. The results indicate that various cytochrome P-450 isozymes of rat liver, which can be induced by pretreatment in vivo with phenobarbital or 3-methylcholanthrene, exhibit different stereoselective properties in catalyzing the epoxidation reactions at the K region of BA and of B(a)P.

Work from many laboratories on hepatic microsomal P-450 from rabbits and rats has established that many different forms of P-450 exist, differing with respect to overall substrate specificity and even regio- or stereoselectivity for the metabolism of individual substrates. The relative proportions and total concentrations of various P-450 isozymes in liver microsomes may be significantly altered following exposure of animals to foreign chemicals. Seven different forms of cytochrome P-450 have been purified from rat liver microsomes (35). The major MC inducible cytochrome P-450 (form c) exhibits its greatest activity toward both B(a)P and 7,12-dimethylbenz(a)anthracene (DMBA) and forms substantially high spin, high affinity complexes ($K_d=10$ nM) with both hydrocarbons. Cytochrome P-450d, a minor MC-inducible form, has far lower activity for metabolism of both polycyclic aromatic hydrocarbons, yet also forms high affinity complexes ($K_d=100$ nM) with both PAHs, retaining the full high spin state of the free cytochrome. Although two phenobarbital (PB)-induced forms (P-450s b and e) differ by only 13 amino acids, they exhibit significant differences in metabolism of PAH and in complex formation. Whereas P-450b is only active in the metabolism of DMBA (9.8 nmol/nmol P-450/min versus 1.9 for BP), P-450e has low activity for both substrates (3.3 and 1.2 nmol/nmol P-450/min). Nevertheless, P-450e forms a high affinity complex ($K_d=100$ nM) with both PAHs that enhances the proportion of the high spin state (from 30% to 70%). P-450s a, and h, and pregnenolone-16-carbonitrile (PCN) exhibit little activity toward B(a)P or DMBA, but P-450 PCN does form a low spin complex with B(a)P (not DMBA). Antibodies to all P-450 forms were prepared and purified. All antibodies exhibited behavior on Ouchterlony plates similar to that previously reported, i.e., both anti-P-450s b and e cross-reacted completely, anti-P-450s c and d showed partial cross-reactivity, and anti-P-450s a, and h, and PCN reacted only with its homologous isozyme.

This group also reports that the metabolism of both B(a)P and 7,8-dihydrodiol by MC-induced rat liver microsomes is subject to severe inhibition by primary metabolites of B(a)P, which was analyzed by determining individual inhibition constants for all primary B(a)P metabolites for both B(a)P and 7,8-dihydrodiol metabolism. Monooxygenation of 7,8-dihydrodiol was, surprisingly, 5 to 10 times more sensitive than monooxygenation of B(a)P to inhibition by all primary metabolites even though both reactions require the same enzyme, cytochrome P-450c. Two representative products, 1,6-quinone and 9-phenol, were both strong, competitive inhibitors of B(a)P metabolism with K_i values of 0.12 and 0.74 micromolar, respectively. The total effect of product inhibition on the overall reactions was determined by fitting progress curves of B(a)P, 7,8-dihydrodiol and anti-7,8-dihydrodiol-9,10-oxide (determined as 7,10/8,9-tetrol) over a range of B(a)P concentrations to integrated steady-state equations using experimental V_{max} and K_m values. The effective product inhibition factors for B(a)P and 7,8-dihydrodiol metabolism, determined from progress curve fits, were only twofold higher than the corresponding calculated theoretical values. The effective product inhibition factors, obtained from progress curve analysis, confirmed that 7,8-dihydrodiol metabolism was substantially more sensitive to inhibition by primary B(a)P metabolites than B(a)P metabolism itself. This difference probably reflects the much

higher affinity of cytochrome P-450c for B(a)P ($K_d=6$ nM), as compared to 7,8-dihydrodiol ($K_d=175$ nM) that was established spectrophotometrically both for the purified cytochrome and for MC microsomes.

Enzymes that increase or decrease the levels of epoxides in the cell should have a major effect on epoxide-mediated carcinogenicity. One such enzyme that affects the intracellular concentration of epoxides is epoxide hydrolase, which converts epoxides to dihydrodiols. This conversion is normally regarded as a deactivation step. For example, epoxide hydrolase has been shown to deactivate the mutagenic epoxide, benzo(a)pyrene-4,5-oxide. This deactivation has been demonstrated both as a decrease in the mutagenicity of this compound in *Salmonella* tester strains and in the epoxide hydrolase-dependent decrease in the covalent binding of this metabolite to DNA. However, in some cases dihydrodiols can be further metabolized to other potent carcinogenic intermediates. Benzo(a)pyrene-7,8-oxide can be converted via epoxide hydrolase to benzo(a)pyrene-7,8-diol, which can in turn be oxidized once again to form the very potent carcinogen benzo(a)pyrene-7,8-diol-9,10-oxide. B(a)P can cause skin and lung cancers in experimental animals. Because epoxides are involved as intermediates in the bioactivation of such a large number of toxins and carcinogens, the levels and substrate specificities of epoxide hydrolases can be regarded as important factors in cellular mechanisms of defense against these chemicals.

Several different epoxide hydrolases have been shown to exist in the cell. These enzymes can be distinguished on the basis of their substrate specificities and their ability to bind to specific antibodies. The most widely studied epoxide hydrolase is a membrane-bound form that catalyzes the hydrolysis of arene oxides and of other *cis*-disubstituted and monosubstituted oxiranes. This epoxide hydrolase actively metabolizes the model substrates, styrene oxide and benzo(a)pyrene-4,5-oxide, and is referred to as benzo(a)pyrene-4,5-oxide hydrolase. The inhibition *in vitro* and induction *in vivo* of microsomal *trans*-stilbene oxide hydrolase have been studied (29). This microsomal epoxide hydrolase activity is distinguishable from the previously well-defined microsomal arene oxide hydrolase by a number of catalytic criteria. Two substituted chalcone oxides, 4-phenylchalcone oxide and 4'-phenylchalcone oxide, are potent inhibitors of microsomal *trans*-stilbene oxide hydrolase, but have no apparent activity against benzo(a)pyrene-4,5-oxide hydrolase. Conversely, compounds which are potent inhibitors of benzo(a)pyrene-4,5-oxide hydrolase, including styrene oxide, cyclohexene oxide, and trichloropropene oxide, inhibit microsomal *trans*-stilbene oxide hydrolase only at very high (millimolar) concentrations. The chalcone oxides inhibit microsomal *trans*-stilbene oxide hydrolase noncompetitively and have nanomolar affinity constants for the enzyme. Attempts were made to induce microsomal *trans*-stilbene oxide hydrolase *in vivo*. Compounds that induced microsomal benzo(a)pyrene-4,5-oxide hydrolase levels in mice did not simultaneously induce *trans*-stilbene oxide hydrolase levels. Clofibrate was an exception; it induced levels of both enzymes to a small but statistically significant degree. The two microsomal hydrolase activities have, therefore, very different catalytic sites and appear to be under separate genetic control. 4-Phenylchalcone oxide and 4'-phenylchalcone oxide are selective inhibitors of microsomal *trans*-stilbene oxide hydrolase and may prove to be very useful in assessing the involvement of this enzyme in the metabolism of endogenous or xenobiotic epoxides.

The ultimate tumorigenic and mutagenic metabolite of B(a)P is 7-beta,8-alpha-dihydroxy-9-alpha,10-alpha-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BaPDE) and its reactions with DNA *in vivo* have been extensively studied. It is known that BaPDE binds physically to double-stranded DNA, most likely by an intercalation

mechanism, prior to reacting covalently with nucleic acid bases (31). This covalent reaction occurs at the 10-position of BaPDE and preferentially involves the exocyclic amino group of guanine. Since guanine is the primary target for BaPDE, studies of the interactions of this diol epoxide with the synthetic polynucleotides, poly(dG-dC)·(dG-dC) and the 5-methylcytosine derivative poly(dG-m⁵dC)·(dG-m⁵dC), are of interest. The influence of conformational effect on the reactivities can be investigated, since these polynucleotides are known to undergo salt concentration-dependent conformational changes from the B form (right-handed helix) to the Z form (left-handed helix). The existence of segments of left-handed helices in living cells has been demonstrated, and these phenomena may thus be important in vivo. The physical and covalent binding of BaPDE to poly(dG-dC)·(dG-dC) and poly(dG-m⁵dC)·(dG-m⁵dC) in the B and Z forms were studied utilizing absorbance, fluorescence and linear dichroism techniques. In the case of poly(dG-dC)·(dG-dC), the decrease in the covalent binding of BaPDE with increasing NaCl concentration (0.1-4 M) as the B form is transformed to the Z form is attributed to the effects of high ionic strengths on the reactivity and physical binding of BaPDE to the polynucleotides; these effects tend to obscure differences in reactivities with B and Z forms of the nucleic acids. In the case of poly(dG-m⁵dC)·(dG-m⁵dC), the B to Z transition is induced at low ionic strength (2 mM NaCl+10 micromolar Co(NH₃)₆Cl₃) and the covalent binding is found to be 2-3 times lower to the Z form than to the B form. Physical binding of BaPDE by intercalation, which precedes the covalent binding reaction, is significantly lower in the Z form than in the B form, thus accounting, in part, for the lower covalent binding. The linear dichroism characteristics of BaPDE covalently bound to the Z and B forms of poly(dG-m⁵dC)·(dG-m⁵dC) are consistent with nonintercalative, probably external, conformations of the aromatic pyrenyl residues.

A chromatographic procedure, originally described by Sawicki et al. for analysis of 7,12-dimethylbenz(a)anthracene:DNA adducts, using boronic acid residues linked to a cellulose support ([N-[N'-[m-[dihydroxyboryl]phenyl]succinamyl]-amino]ethyl-cellulose) was modified to allow the separation of other hydrocarbon-DNA adducts (2). The basic principle of the separation of the syn- and anti-B(a)P metabolite-DNA adducts by boronate chromatography is that the terminal boronic acid group contains two hydroxyls at a spacing that can complex with cis vicinal hydroxyl groups but not with trans. The activation of B(a)P to DNA-binding metabolites in early-passage embryo cell cultures prepared from various species of rodents was investigated by exposing cells from mice (BALB/c and Sencar), rats (Wistar and Fischer 344), and Syrian hamsters to (³H)B(a)P for various lengths of time. The B(a)P:DNA adducts containing cis-vicinal hydroxyl groups such as those formed from 7 beta,8 alpha-dihydroxy-9 alpha,10 alpha-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (anti-BaPDE) were separated from the other types of B(a)P:DNA adducts by immobilized boronate chromatography, and the individual adducts were analyzed by high-performance liquid chromatography. A number of B(a)P:DNA adducts were present in the DNA from the cultures from all three species after 5 h of B(a)P treatment. After a 24-h exposure to B(a)P, the mouse and hamster embryo cell DNA contained a large amount of the adduct formed by reaction of (+)-anti-BaPDE with the 2-amino group of deoxyguanosine (dGuo) and a small amount of a 7 beta,8 alpha-dihydroxy-9 beta,10 beta-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene:dGuo adduct. A large number of B(a)P:DNA adducts derived from 7 beta,8 alpha-dihydroxy-9 beta,10 beta-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene and other unidentified B(a)P metabolites were present in rat embryo cell cultures at all times. Neither the Fischer 344 nor the Wistar rat embryo cell cultures had a significant amount of (+)-anti-BaPDE:dGuo adduct after 5 h of B(a)P treatment, and in the Wistar rat cells larger amounts of other adducts were present even after a 96-h exposure to B(a)P. In cell cultures from all three species, the proportion of (+)-anti-BaPDE:dGuo adduct increased as

the length of time of exposure to B(a)P increased. There are major differences in the metabolic activation of B(a)P to DNA binding metabolites in embryo cells from various species of rodents. However, the variations between cell cultures from different strains of rats or mice are not as great as the variations between cell cultures from different species. The time-dependent alterations in the B(a)P:DNA adducts indicate that analysis after various lengths of time of exposure to B(a)P is essential to characterize, accurately, the pathways of metabolic activation of B(a)P in cells from various species and tissues.

The covalent binding of (+)-anti-BaPDE, the carcinogenic metabolite of benzo(a)-pyrene, and its noncarcinogenic (-) enantiomer to macromolecules was investigated in mouse skin in vivo (11). Levels of the adducts were measured in DNA samples isolated from the epidermis of adult Sencar mice exposed topically to (+)- and (-)-anti-BaPDE for 3, 24, and 72 h. The amount of (+)-anti-BaPDE bound to epidermal DNA was approximately 3 times higher than that of the (-) enantiomer at all time points studied, with the highest level of adducts observed after 3 h exposure. A similar time course of binding was observed in DNA purified from epidermal basal cells which were isolated from mice treated with the two enantiomers. As with the results for isolated DNA samples from whole epidermis, a 3:1 ratio of binding with (+)- and (-)-anti-BaPDE in basal cell DNA was also observed. Interestingly, no significant difference in total binding between the (+) and (-) enantiomers could be detected at any time point in RNA and protein isolated from the basal cells. The formation of individual DNA adducts derived from topically applied (+)- or (-)-anti-BaPDE to mouse skin was monitored at 3, 24, 72 h using high pressure liquid chromatography. The major DNA adduct (64% of total) formed from (+)-anti-BaPDE cochromatographed with marker adducts of N²-(10S-(7R,8S,9R-trihydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene)yl)deoxyguanosine, while other minor adducts also were observed. With the (-)-anti-BaPDE, a greater variety of DNA adducts was formed, with only 20 to 30% of the radioactivity present in high pressure liquid chromatography chromatograms corresponding to the N²-deoxyguanosine adduct. The rate of formation and disappearance of individual adducts derived from both isomers of anti-BaPDE was similar over the 72-h time course. The results suggest that although differences exist in total binding to DNA between the two enantiomers, they do not appear to be of sufficient magnitude to explain the marked difference in biological activity of (+)- and (-)-anti-BPDE in mouse skin. In CD-1 mouse skin the (+)-anti enantiomer was approximately 64 times more active than the (-)-anti enantiomer as a tumor initiator. Similar experiments with Sencar mice, the mouse strain used in the present report, indicated that the (+) enantiomer was approximately 66 times more potent an initiator than was the (-) form.

The rates of formation and disappearance of B(a)P-DNA adducts were analyzed in the epidermis of Sencar mice over a 21-day time course (11). Mice were treated topically with 200 nmol/mouse of (³H)B(a)P at various times prior to sacrifice. The formation and disappearance of total adducts as well as individual adducts was determined and in addition, the rate of DNA turnover was monitored concurrently so that adduct disappearance could be expressed as a function of epidermal cell turnover. Under these experimental conditions, covalent binding of B(a)P to epidermal DNA reached a peak 24 hours after treatment. Interestingly, total B(a)P DNA-adducts disappeared from the epidermis with a biphasic decay curve. Between 24-48 h (Phase A) after application of the hydrocarbon there was a very rapid drop in the level of bound B(a)P to a value greater than 50% of the maximum level at 24 h. Thereafter, the level of bound B(a)P disappeared at a much slower rate (Phase B). In dual-label experiments, where the epidermal DNA was pre-labeled with (¹⁴C)thymidine, (³H)B(a)P-DNA adduct disappearance between 24-28 h was

clearly more rapid than could be explained on the basis of epidermal DNA turnover. By 72 h and beyond, however, (^3H)B(a)P-DNA adduct disappearance approximately paralleled DNA turnover. Examination of the rate of formation and disappearance of individual B(a)P-DNA adducts (nine individual adducts) suggested that some deoxyadenosine (dAdo) adducts were removed more rapidly than deoxyguanosine (dGuo) adducts. Although the anti-BaPDE-dAdo adducts disappeared more rapidly than the major dGuo adduct, this observation alone cannot account for the dramatic drop in total adducts observed between 24-48 h since these represent minor adducts. Thus, it appears that all of the B(a)P-DNA adducts were actively removed during Phase A and the total amount of (+) anti-BaPDE-dGuo adduct removed was much greater than the total amount of dAdo adducts removed during this time period. The results indicate that at least some epidermal cells have the capacity to repair B(a)P-DNA adducts.

Alkylating Agents: Nitrite reacts with amines to produce nitrosamines and with amides to produce nitrosamides. Nitrosamines and nitrosamides constitute the N-nitroso compounds (NNC). Nitrite is usually produced in the environment by the reduction of nitrate. In rats, mice, and hamsters, nitrosamines readily induce tumors of the liver, esophagus, kidneys, nasal cavity and pancreas; and nitrosamides chiefly induce tumors of the glandular stomach, small intestine and nervous and lymphoid systems. The tissue affected depends on the species, the NNC, and the treatment system. There is evidence to suggest that humans are susceptible to NNC carcinogenesis. People could be exposed to NNC either directly or indirectly, via NNC formation in vivo. Direct exposure to NNC occurs in certain industrial situations. Parts/billion (ppb) amounts of NNC are found in some foods and large amounts occur in chewing tobacco, snuff and cigarette smoke. Most in vivo nitrosation probably occurs in the stomach, where gastric acid catalyzes the reaction. When a Western diet is consumed, nitrite concentration in the stomach is normally 120 microgram/liter. Of nitrite entering the stomach, 80% is produced in the saliva by bacterial reduction of salivary nitrate, which, in turn, arises mostly from dietary nitrate. The remaining 20% of nitrite entering the stomach arises from nitrite consumed in the diet, including 8% of total gastric nitrite that originates from nitrite-preserved food products (53).

In another study, the endogenous formation of nitrosoproline (NPRO) following administration of nitrate and proline is reported in ten healthy young adults (79). There was a relatively constant basal excretion of NPRO, 26 ± 10 (SD) nmol/day, in excess of amounts found in the diet. This basal synthesis of NPRO was not reduced by ascorbic acid (2 g/day) or alpha-tocopherol (400 mg/day). A significant rise in the excretion of NPRO was observed following the administration of nitrate and proline, ranging from 29 to 318 nmol/24 h with a mean of 100 nmol/24 h. (^{15}N)Nitrate was used as a tracer to study the observed excess excretion of NPRO in urine. The ingestion of ascorbic acid and alpha-tocopherol inhibited the incorporation of (^{15}N)nitrate into NPRO by 81 and 59%, respectively. An additional nitrosamino acid, N-nitrosothiazolidine-4-carboxylic acid (NTCA), was present in the urine. It was found that NTCA increased sixfold upon ingestion of nitrate. The results of this study show that endogenous nitrosation takes place in humans from both exogenous and endogenous precursors. Nitrosation blocking agents such as ascorbic acid and alpha-tocopherol are clearly effective inhibitors with regard to nitrosation of meal-derived precursors. These experiments show the usefulness of the NPRO test with regard to estimating endogenous nitrosation and reducing this exposure in humans by dietary manipulation. Additionally, measurement of another nitrosamino acid, NTCA, may contribute to the identification of high risk population groups. The measurement of urinary nitrosamino acids in various population groups with different dietary and social

customs can be used to identify the multitude of factors regulating endogenous nitrosation.

NNC could be formed from nitrite and amines or amides as an acid-catalyzed reaction in the stomach and act there to induce gastric cancer (54). Since 5% of ingested nitrate is converted via the saliva to gastric nitrite, a highly significant country-by-country correlation between nitrate intake and gastric cancer incidence in 12 countries supports a role for NNC. Salivary nitrite and nitrate may be inversely related to gastric cancer induction because vegetables contribute heavily to intake of both nitrate and vitamin C/polyphenols, which may counterbalance effects of the nitrate. The Ohshima-Vartsch test for potential gastric NNC formation shows decreased nitrosoproline formation in cases of achlorhydria and increased nitrosoproline formation in smokers and in high-esophageal-cancer areas of China and high-gastric cancer areas of Japan. Gastric cancer association with high-starch diets could be due to a resultant high gastric acidity, which favors nitrosation. Foods associated with gastric cancer (fava beans and dried, salted fish) may supply nitrosatable amines or amides. Other environmental etiologic factors for gastric cancer include high salt consumption, soft water, peaty soil and working in coal mines and oil refineries.

Riboflavin deficiency has been implicated as a contributing factor to human cancers in many epidemiological studies. The association between neoplasms and riboflavin deficiency is also suggested by the correlation between cancer of the upper alimentary tract and Plummer-Vinson syndrome, a disease associated with iron and riboflavin deficiency. One project is studying the effects of riboflavin deficiency on the metabolism of N-nitrosodimethylamine (DMN) and other nitrosamines in rats (88). After weanling rats were put on a riboflavin-deficient diet, the development of the deficiency was monitored by the rate of growth and the erythrocyte glutathione reductase activation coefficient. In riboflavin-deficient rats, the liver microsomal NADPH-cytochrome c reductase activity was lower but the cytochrome P-450 content was higher than that of the control. The metabolism of DMN was dependent on the severity of the deficiency. During mild deficiency, which was observed mainly with Sprague-Dawley rats on this regimen, the microsomal DMN demethylase (DMNd) activity was elevated 30-80%, but the metabolism of N-nitrosomethylbenzylamine and three other nitrosamines was slightly decreased. Dietary restriction in the pair-fed group also caused an elevation of DMNd activity above that of the ad libitum control group due to a partial fasting effect. During severe deficiency, which was observed mainly with Wistar rats, however, the metabolism of DMN, as well as the oxidation of B(a)P, was decreased. Preincubation with flavin adenine dinucleotide and flavin mononucleotide enhanced the DMNd activity of the microsomes from riboflavin-deficient rats but not that from control rats. The results suggest that, depending on the alterations of the monooxygenase enzyme system during the development of the deficiency, riboflavin deficiencies may alter the metabolism of DMN and some other carcinogens.

The metabolism of nitrosamines has been a subject of extensive investigation because of its importance in understanding the carcinogenicity and toxicity of this group of compounds. Oxygenation at the alpha-carbon (alpha-hydroxylation) is believed to be a key step in the metabolic activation of many nitrosamines. There is evidence indicating that N-nitrosodimethylamine (NDMA) is metabolized by a P-450-dependent monooxygenase system. NDMA is one of the most widely occurring carcinogenic compounds. It is a potent carcinogen in many animal species and a suspected carcinogen in human beings. NDMA requires metabolic activation for its chemical interaction with biological macromolecules. The postulated pathway for NDMA activation entails an oxidative demethylation which eventually leads to the

production of a methylating species. It is clear that the alkylation of DNA is the mechanism by which alkylating agents cause mutations. The alkylation of DNA also appears to be a key step in the initiation of chemical carcinogenesis. The metabolism of nitrosamines by microsomal cytochrome P-450 (P-450) isozymes was studied in a reconstituted monooxygenase system (89). P-450 LM₂, LM_{3a}, LM_{3b}, and LM_{3c}, LM₄ and LM₆ were purified, respectively, from the livers of phenobarbital-treated, ethanol-treated, untreated, isosafrole-treated, and imidazole-treated rabbits. Of these isozymes, LM_{3a} had the highest N-nitrosodimethylamine demethylase (NDMAd) activity with a K_m of 2.9 mM and V_{max} of 9.3 nmol/min/nmol. LM₂, LM₄, and LM₆ exhibited NDMAd activity only at high N-nitrosodimethylamine concentrations, and isozymes LM_{3b} and LM_{3c} had poor activity even at the highest substrate concentrations examined. LM₂, however, was more active than LM_{3a} in the metabolism of N-nitrosomethylaniline. With each isozyme (LM_{3a} or LM₄), only one K_m for NDMAd was observed, whereas with rabbit liver microsomes, multiple K_m of 0.07, 0.27, and 36.8 mM were obtained. P-450 isozymes also catalyzed the denitrosation of nitrosamines at rates comparable to or lower than the demethylation, and the ratio of these two reactions was different with different nitrosamines. 2-Phenylethylamine and 3-amino-1,2,4-triazole, which were believed previously to affect NDMAd by mechanisms independent of P-450, were shown to be potent inhibitors of P-450-dependent NDMAd. These results further establish the role of P-450 isozymes in the metabolism of nitrosamines and indicate that LM_{3a} is apparently responsible for the increased N-nitrosodimethylamine metabolism associated with ethanol treatment.

Aromatic Amines: Aromatic amines are thought to induce tumors as a consequence of their N-oxidation and subsequent conversion to reactive metabolites that are capable of altering the structure of nucleic acids. The evidence for metabolic N-oxidation of these structures is extensive, as are the data that show the N-oxidized compounds to be responsible for the modification of nucleic acids of target tissues and to be more effective in producing mutagenic and malignant changes in biological systems. Aromatic amines have been shown conclusively to induce urinary bladder cancer in humans and tumors in a wide variety of organs in experimental animals. The carcinogenic activity of this class of compounds appears to depend on their conversion to reactive metabolites that alter the macromolecules of the target tissues. N-2-Acetylaminofluorene (AAF), one of the most studied aromatic amines, is believed to form DNA adducts following N-oxidation and a subsequent metabolic activation involving conjugation of the hydroxamic acid with sulfate in rat liver, or O-acetylation of the hydroxylamine in the target tissues of several species. Activation by sulfate conjugation can result in the introduction of the AAF moiety at C8 and N2 of guanine in DNA; O-acetylation leads to the introduction of 2-aminofluorene (AF) substituents bound through the arylamine nitrogen to the C8 of guanine. This latter AF structure, which represents the major adduct in all tissue studied thus far, causes much less distortion to the DNA helix than that produced by an AAF adduct (39).

Mammalian peroxidases, found in blood and other tissues, are vital to the body's defense systems and to hormone synthesis. However, because of their strong oxidizing potential, they may also activate foreign compounds to mutagenic and carcinogenic agents. Activation is crucial for N-substituted aromatic compounds which may be industrial and environmental pollutants, model carcinogens or drugs. Peroxidative oxidation of a proximate carcinogen, N-hydroxy-N-2-acetylaminofluorene (N-OH-2AAF), may be via one electron pathway to a nitroxy-free radical, which then dismutates to equimolar N-acetoxy-2AAF and 2-nitrosofluorene (2-NOF), or by a halide-dependent pathway to 2-NOF. Whereas extracts of rat uterus (UT) in cetyltrimethylammonium Br⁻ (Cetab) catalyzed the formation of both products from

N-OH-2AAF, extracts of mammary gland (MG) formed only 2-NOF. At pH 7.4, the ratios of 2-NOF:N-acetoxy-2AAF found with UT extract were 7 and 114, at .004% and .4% Cetab, respectively. This suggests that at the lower Br⁻ concentrations (approximate physiologic levels), both peroxidative pathways occur with UT In vitro, whereas at the higher Br⁻ concentration of one electron oxidation is negligible. At pH 5.5, the latter was also decreased and both UT and MG extracts formed small amounts of 2-nitrofluorene, presumably from 2-NOF. The difference in peroxidative metabolism of N-OH-2AAF by UT and MG suggests the presence of different peroxidase(s) (51).

Oxygen-free radicals have been implicated as etiological agents in several disease processes including aging, arthritis, carcinogenesis, tumor promotion, ischemic damage to tissues and in the toxic action of some drugs. Oxygen-free radicals, in general, are very reactive and are usually present at very low levels, and the fact that these free radicals are not directly observable spectroscopically, even at higher concentrations, has led to difficulties in defining their true role in many biological processes. Thus, there is considerable need for a technique which will aid in probing the role of oxygen-free radicals in biological processes. Products formed by the action of hydroxyl radical attack can be analyzed by the combination of HPLC to separate the products formed and the use of an electrochemical detector with its enhanced sensitivity for detecting the hydroxylation products. The hydroxyl-free radical adduct of deoxyguanosine, 8-hydroxy-deoxyguanosine, can be effectively separated and detected (subpicomoles) by high pressure liquid chromatography with electrochemical detection (LCED). The sensitivity by electrochemical detection is about 1000-fold enhanced over optical detection. Hydroxyl-free radical adducts of salicylate, 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2,5-DHBA) can be detected by LCED at the level of femtomoles. Utilizing adriamycin administration as a model to induce oxygen-free radical tissue damage, the level of DHBAs present in drug-treated rats versus control rats was increased 100-fold in heart and muscle, 30-fold in lung, and three and fourfold in brain and blood, respectively. These first observations support the theory that adriamycin induces hydroxyl radicals in tissues and indicates that the LCED method may prove to be useful to measure oxygen-free radical production in vivo (18).

Major pathways for hepatic metabolism of the hepatocarcinogen, N,N-dimethyl-4-aminoazobenzene (DAB), include N-demethylation, ring hydroxylation, and azo reduction. The metabolic products are excreted in the bile and urine as their respective conjugates with glucuronate, sulfate, and glutathione. Reduction of the azo linkage in drugs and other foreign chemicals has been known for many years. Within the liver, several sources of azoreductase have been reported. DAB azo reduction was induced in microsomes from rats treated with the hypolipidemic drug, clofibrate, whereas oxidative metabolism of the carcinogen was inhibited. In contrast, treatment with nafenopin, another hypolipidemic drug, inhibited microsomal azo reduction of DAB, whereas oxidative pathways were only slightly affected. Both drugs markedly induced microsomal laurate hydroxylation. DAB azo reduction was increased slightly in microsomes from rats treated with beta-naphthoflavone, while treatment with phenobarbital led to partial inhibition. Pretreatment with isosafrole or pregnenolone-16-alpha-carbonitrile did not significantly alter DAB reduction. Metyrapone, added in vitro, inhibited microsomal DAB azo reductase activity only in phenobarbital-treated microsomes, whereas alpha-naphthoflavone inhibited activity in control and all induced microsomes. DAB azoreduction proceeds readily in air and is not sensitive to carbon monoxide. Neither clofibrate nor nafenopin affected NADPH-cytochrome c reductase activity.

It is concluded that clofibrate-induced azoreductase activity is probably attributable to a specific isoform of cytochrome P-450 which can be distinguished from those which catalyze oxidative pathways of DAB or laurate hydroxylation (43).

Hormones: Another important research activity in the area of carcinogenesis mechanism studies is hormonal carcinogenesis. Interest in this area has accelerated in recent years because of many new developments in biochemical, metabolic and molecular studies of steroid and polypeptide hormones. Hormonal carcinogenesis involves the induction of neoplasms, both benign and malignant, by either natural or synthetic hormones. Of these agents, estrogens appear to possess the most potent carcinogenic and/or promotional activity. Estrogens are a class of chemicals that are defined by their biological function rather than by their structure. Exposure to environmental estrogens may take a variety of routes. There are many naturally occurring estrogenic substances in plants, and some fungi produce estrogenic mycotoxins. Another source of estrogens in the environment arise from synthetic processes. In some cases, an environmental estrogen is made and used as a hormone (e.g., DES), while in others, the products are synthesized for nonhormonal purposes but have estrogenic activity (e.g., DDT). During the past year, interest in the study of carcinogenesis of natural and synthetic estrogens used therapeutically in humans has been extended to certain estrogenic mycotoxins and phytoestrogens. While phytoestrogens do not presently have any therapeutic or commercial use, reduced estrogenic mycotoxin derivatives have been shown to be effective in the treatment of postmenopausal syndrome and are widely employed as implants for growth promotion in cattle. These derivatives can be characterized chemically into three classes, the resorcylic acid lactones, coumestans, and isoflavones. The estrogenic activity of these plant and fungal products has been studied extensively. Resorcylic acid lactones are produced by several contaminants in human food grains and animal feeds. Many different animals are susceptible to toxicosis by these compounds. Fetal abnormalities have been attributed to these estrogenic mycotoxins, as well as squamous metaplasia and hyperplasia. Isoflavones, constituents of many forages and feed, are particularly rich in soya beans. Coumestans are present in forages and legumes, such as alfalfa and clover, at relatively low levels. A new pathway for the metabolism of estrogenic mycotoxins and phytoestrogens to catechol intermediates was recently reported. Interestingly, there appears to be an inverse relationship between estrogenic activity and ability of a given estrogenic mycotoxin to form catechols with hamster liver and kidney estrogen hydroxylase. Catechol estrogens have been postulated to be important intermediates in the sequence of events leading to estrogen-induced tumorigenesis. The carcinogenic potential of estrogens is believed to depend on their rates of metabolic conversion to catechol derivatives rather than on hormonal activity. These data provide the first evidence that both estrogenic mycotoxins and phytoestrogens are capable of forming catechol intermediates. This finding suggests a possible unified mechanism for the carcinogenic effect of diverse types of animal and plant estrogens (44,65).

Experiments conducted in many laboratories throughout the world have led to a recognition that steroid hormones, in general, effect their biologic responses in target tissues through the mediation of high affinity, specific binding proteins called receptors, which are present in unique amounts in such hormonally responsive tissues. Previous studies by a number of investigators have described two specific nuclear estrogen binding sites in normal and malignant tissues. Type I sites represent the classical estrogen receptor which is transported from the cytoplasm to the nucleus. Nuclear type II sites appear to be a specific nuclear response to estrogenic hormones which are highly correlated with uterine hypertrophy and hyperplasia. Likewise, nuclear type II sites are present in large

amounts in neoplastic tissues, such as mouse mammary tumors and human breast cancer, and this is consistent with a rapid rate of proliferation in these cell populations. As a part of these studies, detailed measurements of estrogen receptors and type II binding sites in the uteri of neonates exposed to diethylstilbestrol (DES) or estradiol (E_2) were made. These measurements have revealed that the uterine cytosol of 10-day-old rats contains very large quantities of type II binding sites and relatively low amounts of estrogen receptor. Thus, it is possible that neonatal exposure to estrogens and the subsequent adverse effects in the adult as a result of this exposure, are being mediated by type II sites. Type II sites bind estrogens with a lower affinity than the estrogen receptor; however, since there are large quantities of these sites in the neonatal uterus they are probably involved in estrogen action. Type II sites in the adult appear to be bound to a ligand which is not estradiol but a compound which is present in the adult diet. Since neonatal rats do not eat solid food and, hence, may have very low quantities of the type II ligand present, this may explain why they have such high levels of type II sites. It is possible that inappropriate estrogen exposure in the absence of type II ligand alters the normal growth responses in the neonatal rat uterus. This type II ligand has been identified as a bioflavonoid. Since mammary tumors contain reduced amounts of the type II ligand, the hypothesis is that this ligand is a cell growth inhibitor. This unexpected blend of information and results now provide investigators with a tool to explore the mechanisms by which neonatal estrogen exposure causes reproductive tract abnormalities (6).

The lack of adequate methods for the quantitation of androgen receptors prevents the study of some of the processes involved in the mechanism of androgen action and its role in carcinogenesis. A procedure has been developed for the measurement of rat prostatic androgen receptor. The combination of sodium cyanide salt with sucrose and sodium molybdate allowed the exchange of endogenous non-radioactive androgen with radioactive androgen. With this method it is now possible to quantitate not only prostatic androgen receptors bound to androgens in vitro but also hormone-receptor complexes formed in intact animals under the influence of endogenous androgen (87).

The initial observation, by others, that cancer cells possess receptors for the vitamin D_3 hormone, (1,25 dihydroxyvitamin D_3) suggested to another grant-supported investigator that D_3 hormone may be regulating growth and differentiation of malignant cells in a manner similar to estrogens, progesterones, testosterone and glucocorticoids. These studies led to the development of a rapid and specific assay for D_3 hormone receptors in cancer cells. Receptor concentration measurement of cell growth and cell DNA synthesis yielded data that demonstrated that the ability of D_3 hormone to inhibit cancer cell growth depended on the cellular content of D_3 hormone receptors. In addition it was found that D_3 hormone inhibited the proliferation of two breast cancer cell lines. The action of this hormone was less potent in media containing higher calcium concentrations. This indicates that the steroid hormone D_3 may be acting at the cellular level by mechanisms involving extracellular calcium (72).

The regulation of gene expression in eukaryotic cells by estrogens and other steroid hormones involves the interaction of specific intracellular receptor proteins with the genome, resulting in the activation of selected sets of responsive genes. A complementary DNA clone (cDNA) containing the entire translated portion of the messenger RNA for the estrogen receptor from human breast cancer cells was sequenced. This cDNA provided a functional protein (gene product) when introduced into Chinese hamster ovary cells. Amino acid sequence comparisons revealed significant regional homology among the human estrogen receptor, the

human glucocorticoid receptor, and the putative v-erb-A oncogene product. This suggests that steroid receptor genes and the avian erythroblastosis viral oncogene are derived from a common primordial gene. This oncogene is unusual in that it shares the genome of the avian erythroblastosis virus with another oncogene, the v-erb-B. The v-erb-B oncogene was transduced from the gene that encodes the cell surface receptor for epidermal growth factor. This homology of estrogen receptor to an oncogene links up the hormone action with carcinogenesis. The cloning of the cDNA and natural gene for human breast cancer cell estrogen receptor will enable investigators to obtain large quantities of human receptor for detailed analysis of structure, composition and function. In addition, introduction of the cloned cDNA into heterospecific prokaryotic and eukaryotic cells will permit a study of the regulation and organization of these genes (27).

Data continue to accumulate supporting the hypothesis that circulating estrogens may function as promoting agents during the development of cancer in women. Studies were conducted to seek evidence for the hormonal basis of the protective effect of first birth on breast cancer risk. The hormonal levels and the bio-availability of estrogen in premenopausal women were studied. It was found that estrogen levels were significantly lower (22%) in parous women compared to those in nulliparous women. Previous studies by these investigators suggested that the protective effect of first birth on breast cancer risk is mediated, in part, by permanently lowering prolactin levels. The current findings suggest that estrogen metabolism also is a factor (37).

Other Agents: The worldwide incidence of esophageal carcinoma varies greatly, with the highest incidence extending in a band from northern China through the Middle East. Epidemiological studies in China suggest that dietary zinc deficiency and environmental exposure to N-nitrosamine carcinogens, such as N-nitrosomethylbenzylamine (NMBA), are among the factors associated with an increased incidence of esophageal carcinoma in humans (33). Rats maintained on a zinc-deficient diet exhibited an increased incidence of NMBA-induced esophageal carcinoma when compared to rats on a control diet. The increased tumor formation was associated with alteration of the microsomal metabolism of NMBA. Weanling male Sprague-Dawley rats were raised on egg protein diets containing 2.3 ppm zinc (low zinc) or 50 ppm zinc (control zinc). Analysis of tissues revealed a rapid decline in the levels of zinc in serum and esophagi of the animals fed the low-zinc diet. Gastric and hepatic zinc content did not differ significantly between the animals fed the low-zinc diet and the animals fed the control zinc diet, even after 6 weeks. The rate of formation of benzaldehyde from NMBA by esophageal mucosal microsomes prepared from the rats fed the low-zinc diet was nearly tenfold higher than that of the rats fed the control zinc diet. This increased rate of NMBA metabolism by esophageal mucosal microsomes from the zinc-deficient rats may explain the increased incidence of esophageal carcinoma in these animals.

The liver is an active site of heme biosynthesis. In rats, as much as two-thirds of the heme made by the liver is utilized for formation of cytochrome P-450 species, the family of mixed-function oxidases that play an essential role in the metabolism of many endogenous and exogenous compounds, including steroids, drugs and carcinogens. Normally, the process of hepatic heme biosynthesis is tightly regulated. However, a number of genetic and acquired defects interfere with hepatic heme biosynthesis. Such defects may give rise to hepatic porphyrias. Clinical relapse of acute hepatic porphyrias may be precipitated by a variety of unrelated lipid-soluble chemicals. Consumption of commercial alcoholic beverages has also been identified as a risk factor in humans with acute hepatic porphyria. These chemicals, including alcohols in commercial alcoholic beverages, cause

induction of both delta-aminolevulinate synthase (ALA-synthase), the rate-limiting step in heme biosynthesis, and cytochrome P-450 in the liver of experimental animals and humans. Three to five carbon alcohols induced ALA-synthase in cultured chick embryo hepatocytes. A direct correlation was found between the extent of induction of ALA-synthase and P-450, suggesting a common mechanism. Ethanol did not induce ALA-synthase, but decreased the extent of induction of ALA-synthase by 2-propyl-2-isopropylacetamide (PIA) and 3,4,3',4'-tetrachlorobiphenyl. General protein synthesis was unaffected by ethanol, indicating that the effect of ethanol on ALA-synthase is selective. Furthermore, possible induction of ALA-synthase by ethanol may have been counterbalanced by an inhibitory effect. Ethanol or isopentanol caused accumulation of 8- and 7-carboxyl-porphyrins from an exogenous source of ALA, compared to otherwise untreated cells which accumulated mainly protoporphyrin. Piperonyl butoxide, an inhibitor of cytochrome P-450, prevented this effect, indicating a role for cytochrome P-450. The results suggest that 3- to 5-carbon alcohols, particularly isopentanol and isobutanol, the predominant higher chain alcohols in commercial alcoholic beverages, may play a role in precipitating clinical relapses of acute porphyria or exacerbating porphyria cutanea tarda in humans who consume alcoholic beverages (73).

CARCINOGENESIS MECHANISMS

GRANTS ACTIVE DURING FY86

<u>Investigator/Institution/Grant Number</u>	<u>Title</u>
1. ARCHER, Michael C. Ontario Cancer Institute 2 R01 CA26651-07	Mechanism of Nitrosamine Alkylation of DNA and RNA
2. BAIRD, William M. Purdue University West Lafayette 5 R37 CA28825-07	Modifiers of Chemical Carcinogenesis in Cell Culture
3. BHARGAVA, Madhu M. Yeshiva University 5 R01 CA32268-03	Protein Binding in Hepatic Fate of Chemical Carcinogens
4. BUHLER, Donald R. Oregon State University 5 R01 CA22524-08	Pyrrolizidine Alkaloid Toxicity, Metabolism, and Binding
5. CAVALIERI, Ercole L. University of Nebraska Medical Center 5 R01 CA32376-03	Mechanisms of Mammary Carcinogenesis by Hydrocarbons
6. CLARK, James H. Baylor College of Medicine 5 R01 CA26112-05	Effect of Estrogen on Normal and Abnormal Cell Growth
7. COHEN, Samuel M. University of Nebraska Medical Center 5 R01 CA 32313-03	Nonmutational Multistage Urinary Bladder Carcinogenesis
8. COLBY, Howard D. West Virginia University 5 R01 CA 22152-06	Adrenal Carcinogen Metabolism
9. COX, Ray Univ. of Tennessee Ctr. Health Sci. 5 R01 CA15189-12	Ethionine Carcinogenesis
10. CUCHENS, Marvin A. Univ. of Mississippi Medical Center 5 R01 CA33111-03	Carcinogenesis of B-Lymphocytes: Peyer's Patches
11. DIGIOVANNI, John Univ. of Texas System Cancer Center 5 R01 CA36979-03	Role of DNA-Binding in Skin Tumor Initiation
12. EBLEN, William R. Rene Dubos Center for Human Environments 1 R13 CA42728-01	The Rene Dubos Forum Program

13. EL-BAYOUMY, Karam E.
American Health Foundation
5 R01 CA35519-03
Nitroaromatics: Carcinogenicity
and Metabolism
14. ERNSTER, Lars
University of Stockholm
5 R01 CA 26261-06
The Metabolism of Polycyclic
Hydrocarbons and Cancer
15. FANNING, James Collier
Clemson University
5 R01 CA35733-03
The Nitrosation of Amines with
Iron Nitrates
16. FIALA, Emerich S.
Naylor Dana Institute for Disease Prev.
5 R01 CA31012-05
Disposition of Hydrazines: Species
and Strain Effects
17. FLOSS, Heinz G.
Ohio State University
5 R01 CA37661-02
Biochemical Mechanisms of Nitro-
samine Carcinogenesis
18. FLOYD, Robert A.
Oklahoma Medical Research Foundation
5 R01 CA18591-10
Carcinogen-Free Radicals in Aryl
Amine Metabolism
19. FORD, George P.
Southern Methodist University
5 R01 CA38473-02
Prediction of Nucleoside-Carcinogen
Reactivity
20. FRANTZ, Andrew G.
Columbia University (New York)
5 R01 CA 11704-14
Studies on Prolactin and Other
Peptides
21. GESSNER, Teresa
Roswell Park Memorial Institute
5 R01 CA24127-06
Conjugations and Carcinogen
Metabolism
22. GIBSON, David T.
University of Texas (Austin)
5 R01 CA19078-11
Microbial Degradation of Carcino-
genic Hydrocarbons
23. GLUSKER, Jenny P.
Institute for Cancer Research
5 R01 CA10925-36
Application of Crystallographic
Techniques
24. GOLD, Barry I.
University of Nebraska Medical Center
5 R01 CA 24554-06
Epoxidation in Chloroolefin
Carcinogenesis
25. GOLD, Barry I.
University of Nebraska Medical Center
5 R01 CA38976-02
Metabolism and Genotoxicity of
Nitrosamines
26. GOLDMAN, Peter
Harvard University
5 R01 CA34957-04
Carcinogen Metabolism by Host
Intestinal Bacteria

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|---|---|
| 27. GREENE, Geoffrey L.
University of Chicago
5 R01 CA02897-30 | Steroids and Growth |
| 28. GROVER, Philip L.
University of London
5 R01 CA21959-09 | Mechanisms of Activation of
Polycyclic Hydrocarbons |
| 29. GUENTHNER, Thomas M.
University of Illinois at Chicago
2 R01 CA34455-04 | Toxicologic Implications of
Multiple Epoxide Hydrolases |
| 30. HARRINGTON, George W.
Temple University
5 R01 CA18618-11 | Electroanalytical Studies of
N-Nitrosamines |
| 31. HARVEY, Ronald G.
University of Chicago
5 R01 CA36097-03 | Mechanism of Carcinogenesis of
Polycyclic Hydrocarbons |
| 32. HECHT, Stephen S.
American Health Foundation
2 R01 CA32242-04A1 | Carcinogenic Methylated PAH:
Structural Requirements |
| 33. HOLLENBERG, Paul F.
Northwestern University
5 R01 CA16954-10 | Hemoprotein-Catalyzed Oxygenations
of Carcinogens |
| 34. HYLEMON, Phillip B.
Virginia Commonwealth University
5 R01 CA17747-12 | Bile Acids and Large Bowel Carcino-
genesis |
| 35. JEFEOATE, Colin R.
University of Wisconsin (Madison)
5 R01 CA16265-12 | Metabolism of Polycyclic Hydro-
carbons and Carcinogenesis |
| 36. JENSEN, David E.
Temple University
5 R01 CA31503-05 | Chemical Decomposition of Alkylating
Nitroso Compounds |
| 37. JUDD, Howard L.
University of California (Los Angeles)
5 R01 CA23093-08 | Estrogen and Androgen Studies in
Endometrial Cancer |
| 38. KAUFFMAN, Frederick C.
University of Maryland at Baltimore
5 R01 20807-08 | Pharmacology of Carcinogen
Activation in Intact Cells |
| 39. KING, Charles M.
Michigan Cancer Foundation
5 R01 CA23386-09 | Mechanistic Approaches to Carcino-
genesis |
| 40. KOREEDA, Masato
University of Michigan at Ann Arbor
5 R01 CA25185-08 | Bioorganic Chemistry of Arene Oxides
and Related Epoxide |

41. LEE, Mei-Sie
Michigan Cancer Foundation
5 R01 CA37885-02
Metabolic Activation of
Unsubstituted Hydroxamic Acid
42. LEHR, Roland E.
University of Oklahoma (Norman)
5 R01 CA22985-09
Diol Epoxide and other Derivatives
of PAH and Aza-PAH
43. LEVINE, Walter G.
Yeshiva University
5 R01 CA14231-12
Rôle of Metabolism in the Biliary
Excretion of Drugs
44. LI, Jonathan J.
University of Minnesota (Mnpls-St Paul)
5 R01 CA22008-09
Estrogen Carcinogenicity and
Hormone-Dependent Tumors
45. LI, Jonathan J.
University of Minnesota (Mnpls-St Paul)
1 R01 CA41387-01
Sex Hormones and Hepatocellular
Carcinomas
46. LIEHR, Joachim G.
Univ. of Texas Med. Br. (Galveston)
7 R01 CA43233-01
Mechanism of Estrogen-Induced Renal
Carcinogenesis
47. LISTOWSKY, Irving
Yeshiva University
1 R01 CA42448-01
High Affinity, High Capacity
Steroid, and Carcinogen Binding
48. LOEPPKY, Richard N.
University of Missouri (Columbia)
5 R01 CA26914-06
Carcinogenesis: Nitrosamine
Formation and Inhibition
49. LOEPPKY, Richard N.
University of Missouri (Columbia)
5 R01 CA22289-09
Nitrosamino Fragmentation and
Nitrosamine Carcinogenesis
50. MAGEE, Peter N.
Temple University
5 R01 CA23451-08
Formation and Metabolism of
N-Nitroso Compounds
51. MALEJKA-GIGANTI, Danuta
University of Minnesota (Mnpls-St Paul)
5 R01 CA28000-07
Mammary Carcinogenesis by
N-Substituted Aryl Compounds
52. MARCHOK, Ann C.
Oak Ridge National Laboratory
7 R01 CA43857-01
Effects of HCHO and Benzopyrene
in a New Tracheal Model
53. MIRVISH, Sidney S.
University of Nebraska Medical Center
5 R01 CA32192-03
N-Nitroso Compounds Formed In Vivo
from Nitrogen Dioxide
54. MIRVISH, Sidney S.
University of Nebraska Medical Center
5 R01 CA35628-03
Nitrosamine Metabolism in the
Esophagus

55. MUKHTAR, Hasan
Case Western Reserve University
5 R01 CA38028-02
Goeckerman Therapy of Psoriasis--
Oncogenic Mechanisms
56. NAGEL, Donald L.
University of Nebraska Medical Center
5 R01 CA31016-05
An In Vitro Model for Alkylation
by Pancreas Carcinogens
57. NEWMAN, Melvin S.
Ohio State University
5 R01 CA07394-20
Synthesis of Substituted Polycyclic
Hydrocarbons
58. NICOLAOU, Kyriacos C.
University of Pennsylvania
5 R01 CA36196-03
Synthesis and Biology of Unstable
Natural Products
59. OFNER, Peter
Tufts University
5 R01 CA29513-05
Androgens in Prostatic and
Epididymal Culture
60. PAQUETTE, Leo A.
Ohio State University
5 R01 CA12115-15
Unsaturated Polyolefins and
Hydrocarbon Carcinogenesis
61. PARTHASARATHY, Rengachary
Roswell Park Memorial Institute
2 R01 CA23704-07A1
Stereochemistry of Thiol-Disulfide
Interchanges
62. PENNING, Trevor M.
University of Pennsylvania
1 R01 CA39504-01A1
Dihydrodiol Dehydrogenase and the
Aspirin-like Drugs
63. PIETTE, Lawrence H.
Utah State University
7 R01 CA42013-01
ESR Studies of Biological-Free
Radical Mechanisms
64. POUR, Parviz M.
University of Nebraska Medical Center
5 R01 CA35042-03
Prevention of Nasal Cavity Tumors
by Castration
65. PURDY, Robert H.
Southwest Foundation for Biomedical Res.
1 R01 CA41569-01
Role of Catechol Formation in
Estrogen-Mediated Cancer
66. REINKE, Lester A.
Univ. of Oklahoma Hlth. Sci. Ctr.
5 R01 CA30137-06
Influence of Ethanol on Carcinogen
Activation
67. RIGBY, James H
Wayne State University
5 R01 CA36543-02
Synthesis of Cocarcinogenic
Diterpenes
68. ROSEN, Jeffrey M.
Baylor College of Medicine
5 R01 CA16303-11
Hormonal Regulation of Breast
Cancer

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| 69. SCRIBNER, Norma
Pacific Northwest Research Foundation
5 R01 CA23712-08 | Early and Critical Events in
Chemical Carcinogenesis |
| 70. SEGAL, Alvin
New York University
5 R01 CA24124-06 | Carcinogenic Acylating Agents and
Mode of Action |
| 71. SHIMAMURA, Tetsuo X.
Univ. of Medicine and Dentistry of NJ
5 R01 CA30106-03 | Mechanisms of Development of
Urinary Bladder Cancers |
| 72. SIMPSON, Robert U.
University of Michigan at Ann Arbor
5 R23 CA36507-03 | Actions of 1,25 Dihydroxyvitamin
D3 on Malignant Cells |
| 73. SINCLAIR, Peter R.
Dartmouth College
5 R01 CA25012-08 | Liver Cell Cultures for Study of
Carcinogen Activation |
| 74. SINHA, Dilip K.
Roswell Park Memorial Institute
5 R01 CA36139-03 | Protection Against Mammary Carcino-
genesis by Pregnancy |
| 75. SLAGA, Thomas J.
Univ. of Texas System Cancer Center
5 R01 CA34962-03 | Polycyclic Hydrocarbon Metabolism
and Binding in Skin |
| 76. SMITH, Louis C.
Baylor College of Medicine
5 R01 CA31513-03 | Cellular Uptake of Carcinogens |
| 77. STROBEL, Henry W.
Univ. of Texas Hlth. Sci. Ctr. (Houston)
5 R01 CA37148-03 | Colonic Carcinogenesis/Chemotherapy
and GI Hormones |
| 78. SULLIVAN, Paul D.
Ohio University Athens
5 R01 CA34966-03 | Structure and Metabolism of
Substituted Benzo(a)Pyrenes |
| 79. TANNENBAUM, Steven R.
Massachusetts Institute of Technology
2 P01 CA26731-07 | Endogenous Nitrite Carcinogenesis
in Man |
| 80. TAYLOR, John-Stephen A.
Washington University
1 R01 CA40463-01 | DNA Photolesion Structure-Activity
Relationships |
| 81. THURMAN, Ronald G.
Univ. of North Carolina Chapel Hill
5 R01 CA 23080-08 | Pharmacology of Carcinogen
Activation in Intact Cells |
| 82. TOTH, Bela
University of Nebraska Medical Center
2 R01 CA31611-04 | Carcinogenesis and Chemistry of
Cultivated Mushrooms |

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| 83. VESSELINOVITCH, Stan D.
University of Chicago
2 R01 CA25522-07 | Role of Sex Hormones in Hepato-
Carcinogenesis |
| 84. VOLLHARDT, K. Peter
University of California (Berkeley)
5 R01 CA20713-09 | Activated Mutagenic and Aromatic
Hydrocarbons |
| 85. WALKER, Bruce E.
Michigan State University
1 R01 CA41599-01 | Mechanism of DES Transplacental
Carcinogenesis |
| 86. WHALEN, Dale L.
University of Maryland Balt. Co. Campus
5 R01 CA26086-06 | Kinetics of Nucleic Acid-Catalyzed
Epoxide Hydrolyses |
| 87. WOTIZ, Herbert H.
Boston University
5 P01 CA28856-06 | The Role of Hormones and Binding
Proteins in Cancer |
| 88. YANG, Chung S.
Univ. of Medicine & Dentistry of NJ
5 R01 CA16788-10 | Monooxygenase: Properties and
Carcinogen Activation |
| 89. YANG, Chung S.
Univ. of Medicine & Dentistry of NJ
5 R01 CA37037-03 | Enzymology and Mechanisms of
Nitrosamine Metabolism |
| 90. YANG, Shen K.
Uniformed Services Univ. of Hlth. Sci.
2 R01 CA29133-05 | Metabolic Activation of Monomethyl-
benz Anthracenes |

SUMMARY REPORT

DIET AND NUTRITION

The Diet and Nutrition component within the Chemical and Physical Carcinogenesis Branch contains 42 grants with FY86 funding of \$5,459,367 million. The component supports laboratory investigations searching for cancer etiologic factors related to diet and nutrition. These investigations include mechanism studies of cancer induction by a variety of dietary constituents (i.e., fats of varying sources and saturation levels, proteins of various types and levels, fiber, nitroso compounds, mycotoxins and other naturally occurring carcinogens, inhibitors of carcinogenesis, compounds associated with the gut including bile acids/fecal steroids and the influence of microflora). In addition, support is given to studies which focus on specific dietary factors (i.e., nutrients or micronutrients, host factors involved in pathogenesis, and the development of methods or refinements of techniques for identifying putative carcinogens in foods, body fluids or feces, as well as the influence of various methods of food processing and cooking).

Several studies with a focus on dietary fat are currently underway. Murine mammary tumor virus (MuMTV) is known to play a major role in the development of mammary tumors in mice. Dietary calorie and fat have been shown to profoundly influence the time of onset and the incidence of murine mammary tumors. The objectives of one study (29) was to obtain 1) an understanding of how calorie-restricted diets with low and high fat content modulate the development of mammary tumors in mice, and 2) to determine the effect of different diets on mammary tumor metastasis and if there is a relationship between MuMTV expression and tumor metastasis. This study used a number of mouse strains in which either exogenous (strains C3H, RIII and A) or endogenous (strain GR) MuMTV induced mammary tumors. Mice were fed with three different diets beginning at the time they were weaned: 1) high calorie (16 cal/day), low fat (5% corn oil); 2) low calorie (10 cal/day), low fat; and 3) low calorie, high fat (20% corn oil) diets. Histological methods were used to quantitate the development of hyperplastic alveolar lesions (HANs) in their mammary glands. Thin section electron microscopy was used to evaluate the production of MuMTV particles, MuMTV-RNA was quantitated by molecular hybridization, radioimmunoassays were used to quantify serum prolactin and MuMTV proteins, and high pressure liquid chromatography was used to evaluate the fatty acid composition of prostaglandin production by mammary glands and mammary tumors. Organ cultures of whole mammary glands from adult mice were used to study, directly, the effects of fatty acids on the development of HANs and MuMTV expression. The effect of calorie-restricted diets, containing low or high fats, on tumor progression and metastasis was determined by using a transplantable mammary tumor outgrowth line (RIII/NOD).

The results show that a low calorie, low fat diet when compared with a high calorie, low fat diet 1) dramatically decreases the incidence of spontaneously occurring mammary tumors, 2) reduced the growth of HANs, 3) inhibits MuMTV production, and 4) lowers prolactin levels. Also, it was found that the effect of calorie restriction can be overcome by feeding mice with a low calorie, high fat diet and that the progression of HANs, MuMTV production and the level of circulating prolactin in these mice were comparable to that in mice fed ad libitum or a high calorie, low fat diet. On the basis of these observations, it is hypothesized that dietary calories and fat primarily affect the production of prolactin which, in turn, alters the expression of MuMTV. Such changes in MuMTV production

by dietary calories and fat may thus change the probability of MuMTV proviral integration which is important for the transformation of mammary cells and mammary tumor development.

A second experiment addresses the question of whether a high fat diet acts as a promoter for MuMTV-induced mammary tumorigenesis. In preliminary experiments the organ cultures of HAN-containing mammary glands from adult mice were used and the effects of fatty acids and a prostaglandin synthase inhibitor on the survival and/or growth of HAN were examined. It was found that the number of HANs per gland was increased in cultures treated with arachidonic acid and was decreased after treatment with stearic acid or indomethacin. Arachidonic acid also induced higher incorporation of (^3H)thymidine into the cellular DNA of HAN-containing glands. A comparison of (^3H)arachidonic acid uptake into intact (HAN-containing) glands and parenchyma-free fat pads revealed a selectively higher incorporation of the labeled fatty acid in the intact glands. Arachidonic acid exposure produced higher cumulative amounts of prostaglandin E_2 (PGE_2) which was significantly inhibited by indomethacin. In contrast, exposure to stearic acid did not lower PGE_2 levels below that of controls. A concomitant increase in HAN number and PGE_2 production by arachidonic acid and a decrease in both, caused by indomethacin, suggest that arachidonic acid conversion to PGE_2 may have a facilitative role in the survival of HAN. The implication of these results is that polyunsaturated fatty acids in vivo may function in promoting the growth of preneoplastic and neoplastic mammary cells. Further studies are in progress to obtain a better understanding of the mechanism of action of fatty acids on mammary cells.

Other investigations into the effect of diet on the growth of transplantable mammary tumors revealed that the resultant size of tumor implants in mice fed a low calorie, low fat diet for 6 weeks following transplantation is 28% smaller than those in mice fed an isocaloric, high fat diet. In addition, there was about 70% inhibition in the incidence of lung metastasis in the former group of mice when compared with the latter group. However, mice fed an isocaloric, low fat diet 4 weeks prior to, and 6 weeks after, transplantation showed 75% inhibition in tumor size when compared with those mice that were fed an isocaloric, high fat diet for the same period of time. Similarly, mice fed an isocaloric, low fat diet exhibited 90% reduction in the incidence of lung metastasis compared with those mice fed an isocaloric high fat diet. These results demonstrate that restricted intake of calories, in combination with reduction of dietary fat, is more effective in suppressing mammary tumor growth and metastasis than either of these factors alone and that dietary restriction prior to, and after, transplantation further modulates tumor progression. Since diet appears to regulate the metastasis of this particular tumor line, current work using this model system is continuing to determine if diet changes the expression of certain cell surface antigens, including MuMTV glycoproteins, and/or alters the composition of tumor cell membranes which might play an important role in influencing metastasis.

Another study (26) has been conducted with the objective of gaining new insights into the interaction of dietary selenium, vitamin E and type of fat (saturated and polyunsaturated) in colon carcinogenesis. Specifically, the study proposed to: (1) assess the role of dietary excess, adequacy or deficiency of selenium, or dietary excess of vitamin E, individually and in combination, on azoxymethane (AOM)-induced colon carcinogenesis in rats fed the diets containing lard and corn oil and (2) study the effect of these diets on the activities of glutathione peroxidase and glutathione transferase and on the levels of microsomal membrane fatty acids in liver and colonic mucosa. In the first experiment, the effect of the interaction of selenium deficiency, excess vitamin E and type of fat on colon

carcinogenesis induced by AOM was investigated in male F344 rats. The experimental diets, based on a Torula yeast diet and containing 20% tocopheral-stripped corn oil or 20% tocopheral-stripped lard, were as follows: (1) selenium deficient with adequate vitamin E (50 mg/kg diet), (2) selenium deficient with excess vitamin E (750 mg/kg diet), (3) selenium adequate with adequate vitamin E, and (4) selenium adequate with excess vitamin E. The diets were fed to animals before, during, and after the AOM treatment and until termination of the experiment. It was expected that the selenium deficiency would increase and the dietary excess of vitamin E would inhibit colon carcinogenesis. On the contrary, selenium deficiency significantly inhibited the incidence of colon tumors (% animals with tumors) and of adenocarcinomas of the colon and multiplicity of colon adenocarcinomas and adenomas (tumors per animal), whereas excess vitamin E had no effect on colon carcinogenesis. There was no significant interaction between the selenium status and vitamin E, the selenium status and type of fat, vitamin E and type of fat, or among selenium status, vitamin E and type of fat. With respect to biochemical parameters, selenium deficiency significantly inhibited the selenium-dependent glutathione peroxidase in plasma, liver, kidney, and colonic mucosa. Excess of vitamin E and the type of fat had no effect on the enzyme activity. Selenium deficiency significantly increased the glutathione transferase activity of liver, kidney and colonic mucosa in animals fed adequate or excess vitamin E. The results of the present study demonstrate that selenium deficiency does not promote, and excess vitamin E does not inhibit, colon carcinogenesis. A second experiment investigated the effect of organoselenium, namely p-methoxybenzeneselenol (MBS), in colon carcinogenesis when fed during the stage of initiation. Animals were fed the diets containing 0 and 50 ppm MBS 2 weeks before, during and until 1 week after carcinogen (AOM) treatment. The percent of animals with AOM-induced tumors in the colon and kidney was significantly lower in rats fed the MBS diet than those fed the control diet. The colon tumor multiplicity was also inhibited in rats fed the MBS diet. The results of the present study indicate that the dietary MBS inhibited AOM-induced colon and kidney tumors.

A new method for determining the selenium pool size in man is under development (17). Supplementation of the human diet with the trace element of selenium (Se) is being considered because of its suggested anti-carcinogenic properties. Before such a program can be implemented, techniques for precise assessment of body status of this mineral is needed. This is especially crucial for Se because of its proven toxicity and the wide range of Se intake in different parts of the world. At present, there is no satisfactory method for the assessment of Se status in humans. The measurement of glutathione peroxidase has been used to address the issue of Se deficiency. This method is not applicable in North Americans due to their relatively high intake of the mineral. Alternative methods, based on the measurement of selenium concentration in different tissues and body fluids, are, at best, indirect measures and are only satisfactory at the extremes of exposure. When an isotope of Se is given to a subject, it will mix with body Se. Its dilution is a reflection of the size of various pools of Se and their turnover rates. Isotope dilution can be calculated accurately from samples of urine obtained at different time intervals after administration of a label. This technique should provide a sensitive method for the assessment of selenium status applicable to the entire range of Se intake. A new tracer method has been developed based on the concept of stable isotopes which permits measurement of the Se pool size in man using nonradioactive isotopes of Se. The initial results with adult North Americans are very encouraging. One experiment demonstrated the magnitude of the pool size of body Se which had equilibrated at any given time with the administered stable isotope. For example, 5 days after dosing, the pool

size in human subjects was 3.693 mg. After 35 days, the pool size was 8.685 mg. These data were obtained in four subjects who consumed 100 micrograms Se/day, the usual amount consumed by North Americans. A second experiment demonstrated the effect of changing Se intake on pool size in one subject at day 7 after the isotope was administered. This subject consumed a diet providing the usual intake of 100 micrograms/day and the pool size curve was obtained. Following this, the subject consumed the same diet, except that his daily selenium intake was reduced to 10 microgram (similar to low selenium areas of the People's Republic of China where Keshan disease is prevalent). After 30 days on the low selenium intake, the pool size curve was obtained again. The data clearly demonstrated a significant reduction in the subject's selenium pool size for any time after dosing. The ability to carefully monitor Se status of subjects during the course of conducting supplementation trials is critical to the success of such studies. The proposed method appears to fulfill all the necessary requirements for application to human subjects. Work is continuing on refinement of the procedure for potential field trials.

Another ongoing study (33) is designed to analyze the process of liver carcinogenesis in a well-defined model of nutritional deficiency and to gain further insight into the mechanisms of nutritional modifications of cancer development. Choline deficiency leads to a variety of pathological lesions involving virtually every organ of the body due to a decrease in phospholipid and acetylcholine synthesis and in supply of labile methyl groups. The liver is the major organ which has been affected consistently by choline deficiency for the induction of tumors by chemicals. Past work has demonstrated that the cocarcinogenic effect of a choline-deficient (CD) diet on the induction of liver tumors by several carcinogens is primarily mediated through its promoting action. Several agents have been identified which modify the promoting efficacy of a CD diet, and the modifiers were used to analyze the mechanisms of the diet-induced liver tumor promoter. This study revealed that a CD diet induces peroxidative damage of liver microsomal membrane lipids and many modifiers of CD-diet promotion influence the extent of lipid peroxidation. The substitution of fats in a CD diet with polyunsaturated fat and the addition of methapyrilene, an antihistamine drug, to a CD diet enhanced membrane peroxidation and the promoting activity. An antioxidant and hypolipidemic agents, when added to the diet, suppressed both of these effects. In contrast, phenobarbital did not induce membrane lipid peroxidation and its addition to a CD diet inhibited the diet-induced lipid peroxidation though such a combination exerted a stronger promoting action. Thus, the mechanism of liver tumor promotion by a CD diet and by phenobarbital may be different. The studies are being extended to determine what the consequences of lipid peroxidation in the liver by a CD diet will be. A recent experiment on surface membrane insulin receptors of liver cells in rats fed a CD diet demonstrated a decrease in number and an enhanced binding affinity leading to altered responsiveness of liver cells to insulin-mediated glycogen synthesis. The findings suggest that CD diet-induced lipid peroxidation leads to functional alterations of the membrane receptors involved in cell growth control and may thereby exert its promoting action. Work is continuing to evaluate whether the changes in insulin receptors are the common features of all known liver tumor promoters and whether a CD diet and other liver tumor promoters modify the function of other membrane receptors involved in cell growth control.

A Request for Applications (RFA) for studies on Mutagens in Human Foods was published in the latter part of 1984. The purpose of the RFA was to accelerate the development of additional understanding relative to the possible role, fate,

and cancer relevance of known dietary mutagens commonly present in human foods. The response to the RFA consisted of 25 applications, and after the review process was completed, a total of 9 awards was made. Studies have been underway for several months but it is too early to report findings at this time.

DIET AND NUTRITION
GRANTS ACTIVE DURING FY86

<u>Investigator/Institution/Grant Number</u>	<u>Title</u>
1. AYLSWORTH, Charles F. Michigan State University 5 R23 CA36364-02	Dietary Fat--Cell Communication and Breast Tumorigenesis
2. BARCH, David H. University of Illinois at Chicago 1 R23 CA40487-01	Role of Zinc and Ethanol in Esophageal Carcinogenesis
3. BOKKENHEUSER, Victor D. St. Luke's-Roosevelt Inst. for Hlth. Sci. 2 R01 CA25763-10A1	Bacterial Metabolism of Flavonoid Glycosides/Aglycones
4. BURKE, James P. Pennsylvania College of Podiatric Med. 5 R01 CA 32256-03	Relationship of Zinc to Cellular Membrane Composition
5. CAMPBELL, T. Colin Cornell University Ithaca 5 R01 CA34205-03	Dietary Protein and Chemical Carcinogenesis
6. DAVIES, Donald S. University of London 1 R01 CA40895-01	The Metabolic Fate of Mutagenic Amines in Animals and Man
7. ESSIGMANN, John M. Massachusetts Institute of Technology 1 R01 CA40817-01	Biological Effects of Cyclic Nucleic Acid Adducts Formed
8. FELTON, James S. University of California- Lawrence Livermore National Laboratory 1 R01 CA40811-01	Quantification of Cooked-Food Mutagens by Immunoassay
9. FLEMING, Sharon E. University of California (Berkeley) 1 R01 CA40845-01	Fiber Volatile Fatty Acids and Colonic Cell Biology
10. GALIVAN, John H. New York State Department of Health 5 R01 CA34314-02	Vitamin Function in Liver Studied In Vitro
11. GALLAHER, Daniel D. North Dakota State University 1 R01 CA40843-01	Dietary Fiber and Fat in Bile Acid Excretion
12. GHOSHAL, Amiya K. University of Toronto 1 R01 CA41537-01	Diet and Cancer: Choline and Methionine in Liver Cancer

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| 13. GRUBBS, Clinton J.
University of Alabama at Birmingham
5 R01 CA41994-02 | Effect of Alcohol on Chemically-Induced Cancers |
| 14. HAMILTON, Stanley R.
Johns Hopkins University
5 R01 CA29714-05 | Role of Beer and Ethanol in Experimental Colon Cancer |
| 15. HOTCHKISS, Joseph H.
Cornell University (Ithaca)
5 R01 CA40833-02 | Gastric Formation of N-Nitroso Compounds in the Pig |
| 16. IP, Clement C. Y.
Roswell Park Memorial Institute
5 R01 CA27706-06 | Dietary Minerals and Fats in Breast Cancer |
| 17. JANGHORBANI, Morteza
Boston University
5 R01 CA38943-03 | Dietary Bioavailability of Selenium in Man |
| 18. KINGSTON, David G.
Virginia Polytechnic Institute and State University
1 R01 CA40821-01 | Anaerobic Metabolism of Mutagens in Human Foods |
| 19. KNUDSEN, Ib
Nordic Environmental Mutagen Society
1 R13 CA41557-01 | Conference Support: Genetic Toxicology of the Diet |
| 20. LEIGHTON, Terrance J.
University of California (Berkeley)
5 R01 CA 36890-02 | Origin, Distribution, and Control of Mutagens in Wine |
| 21. MIRVISH, Sidney S.
University of Nebraska Medical Center
5 R01 CA30593-03 | Significance of Nitrosoarea Formation from Creatinine |
| 22. NEWBERNE, Paul M.
Boston City Hospital
1 R01 CA40080-01 | Zinc, Nitrosamine and Esophageal Cancer |
| 23. NEWBERNE, Paul M.
Massachusetts Institute of Technology
5 R01 CA26917-05 | Dietary Fat in Colon Carcinogenesis |
| 24. PETERS, John H.
SRI International
1 R01 CA40918-01 | Fecapentaenes: Mechanistic Studies |
| 25. RAPOPORT, Henry
University of California (Berkeley)
1 R01 CA40984-01 | Metabolism and Bioavailability of MeIQ _x from Fried Beef |

26. REDDY, Bandaru S.
American Health Foundation
5 R01 CA36892-03
Macro- and Micronutrient Interaction in Colon Cancer
27. REDDY, Bandaru S.
American Health Foundation
5 R01 CA 37663-02
Mechanism of Dietary Fat Effects in Colon Cancer
28. RUDOLPH, Frederick B.
Rice University
5 R01 CA 14030-13
Regulation of Metabolism by Purene Interconversions
29. SARKAR, Nurul H.
Sloan-Kettering Institute for Cancer Res.
5 R01 CA25679-05
Effect of Diet on Murine Mammary Tumorigenesis
30. SCANLAN, Richard A.
Oregon State University
2 R01 CA25002-15
N-Nitrosamines in Foods
31. SELIVONCHICK, Daniel P.
Oregon State University
5 R01 CA30087-05
Membrane Protein Composition: Cyclopropenoid Fatty Acids
32. SHIBAMOTO, Takayuki
University of California (Davis)
1R01 CA38697-01
Vapor-Phase Mutagens and Carcinogens in Cooked Foods
33. SHINOZUKA, Hisashi
University of Pittsburgh
5 R01 CA26556-07
Diet Modification & Promotion of Liver Carcinogenesis
34. TAYLOR, Robert T.
University of California-
Lawrence Livermore National Laboratory
1 R01 CA40816-01
Genotoxicity of Food-Related 1,2-Dicarbonyl Compounds
35. THANASSI, John W.
University of Vermont and State
Agriculture College
5 R01 CA35878-03
Vitamin B-6 Metabolism in Hepatomas
36. THOMPSON, Henry J.
University of New Hampshire
5 R01 CA28109-06
Nutrition and Mammary Carcinogenesis
37. TOTH, Bela
University of Nebraska Medical Center
1 R01 CA40989-01
Capsaicin: Chemistry, Carcinogenesis, and Mode of Action
38. VISEK, Willard J.
College of Medicine at Peoria
1 R01 CA41707-01
Calorie Consumption and Experimental Colon Cancer

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| 39. WAGNER, Conrad
Federation of American Societies
1 R13 CA42695-01 | FASEB Conference: Folate, B-12
& One Carbon Metabolism |
| 40. WEINDRUCH, Richard H.
University of California (Los Angeles)
5 R01 CA26164-06 | Dietary Restriction Cancer and
Immune Functions |
| 41. WEISBURGER, John H.
American Health Foundation
2 R01 CA 24217-07A1 | Food Mutagens: Bioassay for
Carcinogenicity |
| 42. WEISBURGER, John H.
American Health Foundation
1 R01 CA42381-01 | Metabolism of the Carcinogen
Aminomethylimidazoquinoline |
| 43. WELSCH, Clifford W.
Michigan State University
1 R01 CA 37613-01 | Caffine and Experimental Mammary
Gland Tumorigenesis |
| 44. WU, Reen
University of California (Davis)
1 R01 CA42097-01 | Retinoids and the Growth of
Respiratory Tract Epithelium |
| 45. WYNDER, Ernst L.
American Health Foundation
1 R13 CA41445-01 | Workshop on Dietary Fat and Fiber
in Carcinogenesis |

SUMMARY REPORT

MOLECULAR CARCINOGENESIS

The Molecular Carcinogenesis component of the Branch includes 256 grants with FY86 funding of approximately \$32.56 million. There are no contracts in this area. The currently active grants consist of 235 R01 (Research Project) grants, 10 R23 (Young Investigator) grants, 6 P01 (Program Project) grants, and 2 R35 Outstanding Investigator grants. In addition, three of the grantees in this component have been selected for the recently approved MERIT award. Research supported by this component focuses on the characterization of carcinogen-macromolecular interactions (2 grants), changes in biological macromolecules and cell functions as a result of carcinogen or cocarcinogen exposure (27 grants), the identification of biochemical and molecular markers and properties of cells transformed by carcinogens (33 grants), the genetics and mechanisms of cell transformation (38 grants), the development of carcinogenicity/mutagenicity testing procedures (20 grants), the mechanisms of carcinogen-induced mutagenesis and genetic damage (19 grants), the identification and properties of tumor promoters and mechanisms of tumor promotion (51 grants), interspecies comparisons in carcinogenesis (4 grants), the genetics and regulation of enzymes associated with carcinogenesis induced by chemical and physical carcinogens (12 grants), development of analytical methodology for detecting chemical carcinogenesis in body fluids and environmental samples (4 grants), and the role of DNA repair in carcinogenesis (27 grants). Expanded descriptions of individual subject areas, along with examples of research accomplishments, are provided below. In January 1986 a Program Announcement entitled "Biological Role of Exocyclic Nucleic Acid Derivatives in Carcinogenesis" was issued. The purpose of this Announcement was to stimulate basic research on a class of important compounds which all have the capability of forming exocyclic nucleic acid adducts. It is expected that studies relevant to many of the above listed subject areas will be stimulated by this program initiative.

Grants Activity Summary

Carcinogen-Macromolecular Interactions: The projects in this subject area focus on studies on the identification, quantitation and characterization of carcinogen-nucleic acid adducts. Interest in the identification and characterization of DNA adducts stems from the role that alterations in DNA play in the initiation of carcinogenesis. Most of the carcinogens used in these studies are metabolized by cellular xenobiotic metabolizing enzymes to a variety of metabolites of which one or a few are reactive and bind to nucleic acids and/or proteins. The identification and quantitation of the binding species are generally determined by chromatographic and radioisotopic techniques. RIA and ELISA assays are increasingly being used with the development of monoclonal antibodies to various carcinogen-nucleic acid adducts. In addition, a technique, using fluorescence line narrowing spectrometry, is being developed that has the potential for analyzing complex mixtures of DNA adducts at a detection level of about five adducts per 10^6 bases (225).

The levels and persistence of specific DNA adducts are often related to the organ specificity of the carcinogen and may indicate which of the adducts are biologically relevant. For many carcinogens, such as the polycyclic aromatic hydrocarbons, alkyl nitrosamines, N-2-acetylaminofluorene, and aflatoxin B₁, the reactive metabolites and the identity of the various nucleoside adducts are known. The chemical nature and physical conformation of the adducts is thought to

determine the biological effect of the adduct. For this reason, several investigators are focusing on the chemical and biophysical characterization of carcinogen-DNA adducts and on the resultant conformational changes the adducts may introduce into the DNA molecule. In many of the studies, defined polydeoxynucleotide sequences containing a modified base are synthesized for analysis. Several different techniques have been utilized for the characterization of carcinogen-nucleic acid adducts. These include high pressure liquid chromatography, absorption and fluorescence spectroscopy, nuclear magnetic resonance, optically detected magnetic resonance, linear and circular dichroism spectroscopy and x-ray crystallography. In addition, computer analysis of possible carcinogen-DNA adduct conformations has allowed the building of molecular models for the most likely conformations. Another determinant of the biological effect of carcinogen DNA adducts is their potential site or sequence-specific interaction on the DNA molecule. The examination of this possibility for aromatic amine, polycyclic aromatic hydrocarbon and the metal carcinogens is the focus of several studies. The development and use of sophisticated molecular biological techniques to analyze site-specific interactions of carcinogens has made this a growing area of interest. The results of these studies give information as to the possible mechanisms by which a carcinogen may cause a mutation or other alteration in the DNA structure.

Alpha, beta-unsaturated carbonyl compounds, such as acrolein, crotonaldehyde and methylvinyl ketone, are ubiquitous in the environment and occur in a wide range of natural and commercial products. These compounds have been shown to be mutagenic in bacteria in the absence of an activating system. Recently, crotonaldehyde was shown to induce liver tumors in F344 rats. In a comparison with N-nitrosopyrrolidine, it appeared to be a weaker tumorigen. Crotonaldehyde has also been shown to readily modify deoxyguanosine in DNA under physiological conditions without metabolic activation, forming cyclic 1,N²-propanodeoxyguanosine adducts. These adducts were also formed from reactions of alpha-acetoxy-N-nitrosopyrrolidine or 4-(carboxynitrosamino)butanal with deoxyguanosine in the presence of esterase. These two compounds are stable precursors of the reactive intermediate generated by alpha-hydroxylation of N-nitrosopyrrolidine. Other alpha, beta-unsaturated carbonyl and related compounds which also form the 1,N²-cyclic guanine adducts include glyoxal, methyl glyoxal, malondialdehyde and alpha-substituted malondialdehydes. These types of compounds are widely distributed in nature and can also be formed endogenously. The demonstration of the tumorigenicity of crotonaldehyde suggests that alpha, beta-unsaturated carbonyl compounds may be an important class of potential carcinogens which warrant further study (90).

While there are numerous studies of the binding of carcinogens to DNA, the interaction of carcinogens with protein may also play a role in their biological effects. This is the hypothesis that is being tested in the laboratory of Dr. Michael C. MacLeod (142). The covalent binding of two stereoisomeric benzo(a)pyrene-7,8-diol-9,10-epoxides (BPDE) to histones was compared in one study. The binding of the anti isomer of BPDE to the core histone fraction was found to be higher than that of the syn isomer. Alternatively, the binding of the syn isomer was higher in the H1 histone fraction. By using reverse-phase high performance liquid chromatography to analyze BPDE binding to histones, it was shown that histone H2A was more heavily labeled by (³H) anti-BPDE and histone H1 by (³H) syn-BPDE. The binding ratio of anti-BPDE as compared to syn-BPDE was even higher for the histone H2A.2 variant than for other histone H2A variants and other core histones. These results show that the BPDE isomers have differential binding

affinities to histones. The covalent binding of anti-BPDE to histone H2A (especially the H2A.2 variant) is suggested to be possibly important in the potential mechanism of action of the carcinogen in nuclei.

There is much interest in determining the influence of DNA sequence on carcinogen binding. A photochemical method has recently been developed which allows the mapping of covalent (+)-anti-benzo(a)pyrene-7,8-diol-9,10-epoxide (anti-BPDE) binding sites within cloned eukaryotic DNA fragments. The method takes advantage of the fact that anti-BPDE adducts contain an intact pyrene chromophore which absorbs light maximally at 346 nm. When anti-BPDE-modified DNA is irradiated with laser light at 355 nm, single-strand cuts are produced in the DNA quantitatively at each modified residue. The cleavage products are then electrophoresed in denaturing agarose gels, the positions of anti-BPDE binding sites within the gene sequence being determined from the size of the resulting cleavage products. This method has been used to examine anti-BPDE binding within the 5' transcriptional control region of the adult chicken beta-globin gene at single base pair resolution. It was found that anti-BPDE binding at individual guanine residues in this region is influenced by nearest neighbor interactions. Binding affinity is at least threefold greater to guanine residues in polyguanine tracts that are greater than or equal to four base pairs long than with nonguanine nearest neighbors. The findings suggest that long poly (deoxyguanine) sequences should be preferred sites for anti-BPDE action in other genes as well (100).

Changes in Cellular Macromolecules and in Cell Functions: The types of research activities in this subject area include studies on alterations in the composition and amounts of various proteins and small molecules, and changes in the pattern of DNA methylation in cells induced by carcinogens to the preneoplastic or neoplastic state. Biochemical and immunochemical methods have been used to isolate, identify and characterize nonhistone chromosomal proteins, phosphoproteins, and cytosolic proteins which are either altered or specifically appear in chemically induced hepatocarcinogenesis models. Neoplastic cells have been shown to manifest a variety of morphological and biochemical phenotypes, different from their normal cell counterparts, which are presumed to result from a substantial reprogramming of the cellular genome during neoplastic transformation. Not all of this reprogramming is thought to be due to direct alterations of the DNA genome. It has been hypothesized that non-DNA factors, so called "epigenetic" effects, play a role in the eventual appearance of neoplastically transformed cells. One possible manner in which the derepression and repression of genes could occur is by alterations in nuclear DNA-nuclear protein complexes. There is also much evidence showing that the state of DNA methylation regulates gene expression and also is involved in the control of cell differentiation. Thus, a greater understanding of the effects of chemical carcinogens and other oncogenic agents on production of aberrant DNA methylation patterns during carcinogenesis is warranted. Several studies are being supported which seek to define the role of altered chromosomal protein-DNA complexes in carcinogenesis and to understand the role of DNA methylation in the control of gene expression and carcinogenesis. Some of the latter studies are focused on elucidating the properties and regulation of DNA methyltransferase, the enzyme responsible for the postreplication methylation of cytosine residues in DNA. Other studies are focused on the state of methylation of specific DNA sequences or genes as a result of carcinogen exposure. The biological effects of DNA hypomethylation, i.e., altered cell differentiation or induction of cell transformation, is being studied by using compounds such as 5-azacytidine, which are known to affect the transfer of methyl groups to DNA.

One rapid cellular response to DNA damage that has been shown to occur is the conversion of NAD to the chromosomal polymer, poly (ADP-ribose). The common factor of DNA damage that stimulates this response is the occurrence of a DNA strand break. Inhibitors of ADP-ribosylation reactions have been shown to strongly potentiate the cytotoxicity of DNA alkylating agents, indicating that ADP-ribosylation reactions are required for cellular recovery from the cytotoxic effects of these agents. The presence of these inhibitors following DNA damage also results in an increased number of DNA strand breaks. In order to understand the mechanism by which inhibitors of ADP-ribosylation block recovery of cell division potential in DNA-damaged, dividing cells, cell cycle progression following DNA damage by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) exposure was studied in the presence of 3-methoxybenzamide (MBA), a potent inhibitor of ADP-ribosylation reactions. Following a dose of 6.8 micromolar MNNG, the presence of MBA was shown to result in an increased length of S phase from about 6.5 h to 10 h and in an accumulation of cells in G₂ with a mitosis delay of 12 h. Progression to the next S phase occurred 5-10 times more slowly and the cells ultimately accumulated in G₂. Increasing the dose of MNNG resulted in a complete block in cell division in the absence of ADP-ribosylation. The results of these studies suggest that ADP-ribosylation reactions, which do not seem to be necessary for DNA excision repair in nondividing cells, appear to be essential for coordinating the events of DNA excision repair with DNA replication and events related to progression through the cell cycle (110).

Approximately 3 to 6% of cytosine residues in the DNA of all vertebrates is modified to 5-methylcytosine, which is predominately found in the dinucleoside sequence 5'-CpG. Substantial evidence has accumulated over the past several years that the methylation of cytosine residues in vertebrate DNA is implicated in the control of gene expression and alterations in cellular phenotype. The mechanisms responsible for copying DNA methylation patterns are not as reliable as those which replicate the DNA base sequence. The fidelity of DNA methylation has been shown to be about 95%, and this lack of complete fidelity may account for the interclonal variations in methylation patterns demonstrated in cultured human diploid fibroblasts. The establishment of methylation patterns in cells has been hypothesized to be dependent on a cell's "methylation capacity" which is defined as the intracellular level of DNA methylase activity divided by the rate of methyltransferase turnover. In order to test this model in eukaryotic systems, the relationship between the amount of extractable maintenance methylase activity and tumorigenic phenotype was studied in nine tumorigenic and nine nontumorigenic cell lines. The results obtained using two different methods showed that, in all but two cases, the extractable nuclear methyltransferase activity was 4- to 3,000-fold higher in tumorigenic than in nontumorigenic cells. Also, only tumorigenic cell lines were shown to have detectable levels of de novo methylase activity. No correlation between levels of DNA methyltransferase activity and 5-methylcytosine content were observed, however. The increased levels of DNA methyltransferase may then give rise to qualitative, but not necessarily quantitative, changes in DNA methylation patterns (113).

In another study, the DNA methyltransferases from normal rat liver nuclei and transplantable hepatocellular carcinoma 252 (THC 252), a chemically induced liver cancer, were characterized and compared. Previous reports from this laboratory had shown that there were no significant differences in the activity or amount of normal and tumor DNA methyltransferases present in either rat or mouse liver. Since the THC 252 genome was shown to be about one-third less methylated than normal liver and there is a hypothesized causal link between alterations in DNA methylation and carcinogenesis, it was of interest to determine if aberrant DNA

methyltransferase species or other isozymic forms, other than the normal one, could be detected in the tumor and to test for any alteration in catalytic response. DNA methyltransferase was purified 700- and 1002-fold from normal rat liver and THC 252 nuclei, respectively. It was found that both tissues yielded a single, major DNA methyltransferase activity that was, as far as could be determined, physically and catalytically indistinguishable. It is thus likely that extrinsic factors are responsible for causing an aberrant or dysfunctional regulation of the methylation reaction during carcinogenesis. Possible mechanisms could be the perturbation of the methylation reaction during either replication or repair by the covalent binding of carcinogens to DNA, or by a direct inactivation of the DNA methyltransferase which could lead to an anomalous inheritance of a cell lineage specific genomic methylation pattern. In one test of the possible mechanisms, it was shown that UVB (280-340 nm)-induced pyrimidine photodimers in an M13 mp9 DNA substrate inhibited highly purified DNA methyltransferase activity in a dose-dependent fashion. For approximately the same number of pyrimidine cyclobutane photoproducts induced, de novo methylation activity was shown to be about twofold more sensitive than maintenance methylation activity. The ability of the pyrimidine photoproducts to inhibit DNA methylation suggests a common mechanism of action with several chemical carcinogens that are known to modify bases (10,132).

There is epidemiological evidence for nickel as a human respiratory carcinogen as well as evidence that certain nickel compounds are carcinogens in animals. Both water insoluble crystalline compounds, such as Ni_3S_2 , and water soluble nickel compounds, such as NiSO_4 and NiCl_2 , were shown to induce transformation of cells in culture with the water insoluble nickel compounds being the more potent transforming agents. Considerable evidence currently exists to demonstrate that nickel ions can interact with and damage DNA. In one study both insoluble (crystalline NiS) and soluble (NiCl_2) nickel compounds were shown to induce single strand breaks and DNA protein cross-links. The single strand breaks were shown to be repaired relatively quickly, but the DNA-protein cross-links were present and still accumulating 24 h after exposure to nickel. Single strand breakage occurred at both noncytotoxic and cytotoxic concentrations of nickel; however, DNA-protein cross-linking was shown to be absent when cells were exposed to toxic nickel levels. The concentration of nickel that induced DNA-protein cross-linking correlated with those metal concentrations that reversibly inhibited cellular replication. In a further characterization of the DNA-protein cross-links induced by NiCl_2 , it was shown that the formation was both time- and concentration-dependent and preferentially occurred in cells in the late S phase of the cell cycle. DNA-protein cross-link formation could not be detected in confluent noncycling cells. The nickel cross-linked proteins were shown to be predominantly associated with magnesium-insoluble regions of fractionated chromatin and include nonhistone chromatin proteins, nonhistone DNA-binding proteins, and a 30,000 MW protein(s) that comigrates electrophoretically with histone H1. Nickel, therefore, appeared to cause the cross-linking of proteins that normally reside in close association with DNA. It is hypothesized that alterations of the normal association of these proteins with DNA may be an early event in the nickel transformation process (35).

The exposure of cells to carcinogens directly affects DNA replication, RNA transcription and RNA transport from the nucleus to the cytoplasm. Several investigators are studying the mechanism of DNA replication following carcinogen-induced DNA damage. Other studies are focused on the characterization of the effects of carcinogen-modified DNA on RNA transcription and the mechanism of altered gene transcription and translation. A possible effect of carcinogen exposure is to alter the fidelity of DNA replication. The identification of cellular factors

which control the fidelity of DNA synthesis, such as altered DNA polymerases, is being explored, as well as the relationship between tumor progression and the fidelity of DNA replication.

Manganese has been shown to be mutagenic in vivo and in vitro with a variety of enzymes and templates and has also been reported to be carcinogenic. Substitution of Mg^{2+} by Mn^{2+} has been shown to reduce the fidelity of DNA synthesis with poly d(A-T) and natural DNA templates and with a variety of DNA synthesizing enzymes. A mechanistic explanation for these effects is still lacking. In this study the mechanism of manganese mutagenesis was analyzed using *E. coli* DNA polymerase I with poly d(A-T) and phi X174 DNA templates by determining the dependence of error rate on free Mn^{2+} concentration and comparing this to measured dissociation constants of Mn^{2+} from enzyme, template and deoxynucleoside triphosphate substrates. From this comparison several conclusions were reached. At very low Mn^{2+} concentrations, the enzyme is activated at high fidelity. It was considered unlikely that activation with manganese per se significantly alters the conformation of enzyme so as to affect nucleotide selection. At low, free Mn^{2+} concentrations of less than 100 micromolar, manganese was shown to cause errors in incorporation via its interaction with the DNA template. The concentration dependence of mutagenesis is determined by the strength of binding Mn^{2+} to the particular DNA template used. The data obtained do not allow the ruling out of the possibility that Mn^{2+} -deoxynucleoside triphosphate interactions contribute to mutagenesis in selected situations. This range of free Mn^{2+} concentrations is the one of greatest relevance for in vivo mutagenesis. At higher concentrations of between 0.5-1.5 millimolar, further mutagenesis by Mn^{2+} was shown to occur. This mutagenesis is thought to be due either to the binding of Mn^{2+} to single stranded regions within the DNA or to weak accessory sites on the enzyme. It is implied that other metal ions also reduce fidelity by interaction with DNA and that this is the mechanism of metal-induced mutagenesis. However, since one exception (Be^{2+}) is known, the mechanism for metal-induced infidelity involving metal DNA interactions will have to be substantiated in each individual case (139).

Markers and Properties of Transformed Cells: Research included in this subject area involves studies on the documentation of various growth and functional properties of initiated cells, preneoplastic cells and fully transformed cells, and the identification of biochemical and molecular markers for distinguishing these altered cell types from normal cells. The evidence obtained to date strengthens the supposition that the development of most cancers involves a multi-step process in which cells progress from normal to initiated, preneoplastic, and premalignant stages to the end point of malignant neoplasia. In order to characterize cells at each stage, a detailed analysis and knowledge of the sequence of relevant biochemical and biological alterations associated with the development of chemically induced carcinogenesis is needed. To achieve this purpose, a variety of model systems, both in vivo in animals and in cells in culture, are being used. Of the animal model systems, the predominant one currently in use is the rat chemically induced hepatocarcinogenesis model. Although this model was established some time ago, the treatment regimens being employed have undergone a variety of changes depending on the purpose of the experiment and on the end point desired. Chronic or intermittent exposure regimens have been used, along with initiation-promotion type regimens, in which various initiating carcinogens and promoting stimuli are used. The sequential appearance of foci of altered hepatocytes, nodules, and hepatocellular carcinomas can be observed and analyzed. There are other interesting model systems which are being established and analyzed by one or more laboratories. For example, one interesting experimental system

involves the establishment and sequential analysis of stages of oral carcinogenesis using hamster buccal pouch epithelium. The buccal pouch consists of a flat epithelium which has no glandular elements and normally lacks histochemical evidence of gamma glutamyltranspeptidase (GGT) activity. Whole-mounts of this epithelium can be prepared for analysis. Also, with this system it appears that it will be possible to relate the cells displaying altered growth in vitro to populations of presumptive initiation sites in vivo. This is not possible with other existing models.

Research relevant to respiratory carcinogenesis is being conducted using a rat tracheal implant system. The properties of carcinogen-initiated cells can be studied in short-term organ culture where normal tissue interactions can be preserved. The cells can also be studied while growing in cell culture and also in vivo by allowing the cells to repopulate denuded trachea which are implanted into nude mice. Properties of normal and carcinogen-treated human respiratory epithelium can also be studied by using denuded rat tracheal implants in nude mice. These types of studies are ongoing and represent exciting new approaches to studying respiratory neoplasia and human respiratory neoplasia in particular. It should allow us to better extrapolate animal carcinogenesis results to their human counterpart. Research using other animal model systems, i.e., breast, colon, pancreas, bladder, and prostate, is being handled primarily by the Organ Systems Program of NCI, although some of these model systems are being used in projects supported by this program.

In addition to the utilization of animal systems, the in vitro transformation of cells in culture occupies the focus of several other research groups. The use of cell cultures which are derived from in vivo carcinogenic lesions allows investigators to analyze, more easily, properties of the cells in question. The ability to transform cells in culture allows for the study of mechanistic questions regarding chemically induced transformation. For some of this research, standard rodent fibroblast or epithelial cell lines have been used. With the increasing success in transforming human fibroblast and epithelial cells following the pioneering work of Kakunaga, Milo, and DiPaolo, several groups of investigators are increasingly turning to the use of human cell cultures in their research. This focus has been, and will continue to be, vigorously supported by this program.

Upon transformation by chemicals, most cells acquire altered growth properties which allow them to proliferate under selective growth conditions. This can involve the ability to grow in soft agar (anchorage-independent growth), the loss of contact inhibition of growth, or the ability to grow in medium containing low calcium. Several biochemical and molecular markers have been used to identify transformed, preneoplastic and neoplastic cells. The histochemical expression of GGT activity and the loss of histochemically determined glucose-6-phosphatase and ATPase activity are common markers used to identify carcinogen-altered liver cells and other epithelial cells. Other enzyme markers, such as the presence of epoxide hydrolase, alkaline phosphatase isozymes and aldehyde dehydrogenase isozymes, are being evaluated. Functional markers for liver cells being utilized currently include the production of albumin, alpha fetoprotein, transferrin, and fibrinogen. An increasing need is being seen for the development of genetic markers of neoplasia. The development of chromosomal abnormalities and aneuploidy in transformed cells are now being evaluated.

Oncomodulin is a small calcium binding protein (approximate molecular weight 11,500) which has been shown to appear in transformed tissues of diverse organs in

various rodents and in humans. It is a member of the troponin C superfamily and shows homology with a group of calcium binding proteins which includes parvalbumin, calmodulin, troponin C and vitamin D-induced intestinal calcium-binding protein. The failure to detect oncomodulin in any normal, untransformed tissue of adult rat suggested that this protein might be oncodevelopmental. Specific antisera to purified rat hepatoma oncomodulin was used to detect oncomodulin in tissues by radioimmunoassay (RIA) and by avidin-biotin-peroxidase complex (ABC) immunohistochemistry. Oncomodulin was detected in placentas increasing from below the limits of detection on day 13 to 25 ng/mg on day 16 of pregnancy and it remained high until the end of gestation. Immunohistochemically, the greatest concentration occurred in the outer placenta and in the parietal and visceral yolk sac and amnion, but not in fetal organs. This occurrence in developing and transformed tissues demonstrates that oncomodulin is an oncodevelopmental protein. In additional studies, human and rat oncomodulins from placentas were purified and characterized. They were shown to be identical to each other and to tumor oncomodulin with respect to amino acid composition, chromatographic elution and pattern of peptides released by digestion with trypsin. This protein has been localized to the cytotrophoblast of the human placenta and mainly in the spongiotrophoblast of the rat placenta, but has been found nowhere else in the fetal or adult rat. The restriction of oncomodulin to extraembryonic tissue (which is the site of expression of several oncogenes), its wide distribution among tumors of adult humans and rodents, and its independence from proliferative stimuli, suggest that oncomodulin is associated with some property which cytotrophoblast cells share with many tumor cells (143).

During chemical hepatocarcinogenesis in the rat, the emergence of hepatocellular carcinomas is preceded by the sequential development in the liver of distinct focal and hyperplastic lesions of altered hepatocytic populations. It is generally accepted that altered hepatic foci (AHF), many of which are morphologically and histochemically distinct from surrounding parenchyma, represent potential precursors of hepatocellular carcinoma in the rat. Both AHF and hepatocellular carcinomas have been found to exhibit similar qualitative alterations in a number of specific enzymatic, antigenic, and functional markers and to demonstrate phenotypic heterogeneity with respect to marker alterations. Studies directed toward the kinetics of appearance and the stability of the cellular changes within the carcinogen-induced AHF provide a means of understanding the nature of the relationship between the AHF and the stages of neoplastic development. In one study the stability and response of histochemical phenotypes of AHF were examined both in the presence and following the withdrawal of 0.05% phenobarbital treatment in rats given a single dose of dimethylnitrosamine 20 to 24 h following partial hepatectomy. The phenotypes of the cells making up the AHF were histochemically scored for the expression of three biochemical markers, gamma-glutamyl transpeptidase (GGT), adenosine triphosphatase, and glucose-6-phosphatase. The AHF exhibited significant heterogeneity with respect to the marker alterations. In the regimen employed, the GGT marker alone scored the great majority of the AHF detected by all three markers. This does not imply that GGT is the most prevalent marker in all hepatocarcinogenic model systems, since AHF or hepatocellular carcinomas induced by peroxisomal proliferating agents do not show the induction of GGT. The results obtained suggest that individual AHF remain phenotypically stable throughout the phenobarbital promotion phase. The presence of GGT may provide a selective advantage for a cell subjected to toxic stress, and thus confer enhanced survival to GGT-positive hepatocytes during chemically induced hepatocarcinogenesis. Further analysis of marker phenotypes of AHF and later lesions may extend our understanding of the role of these

markers in hepatocarcinogenesis and provide insight into the AHF/carcinoma relationship (157).

Gamma-glutamyl transpeptidase (GGT) is a membrane-associated enzyme that is thought to function in the catabolism of glutathione and in absorption or secretion of amino acids across cell membranes. Since markedly elevated levels of GGT activity have been demonstrated in chronically induced neoplasms in rodents, the possibility was raised that GGT might be a useful marker for carcinomas and precancerous lesions in humans. In one study surgical specimens of squamous cell carcinomas and grossly nontumorous squamous mucosa from the oral cavity, oropharynx and larynx from 46 patients were examined histochemically for the presence of GGT activity. Generalized or patchy GGT activity was observed in each of 22 squamous cell carcinomas and in each of 33 dysplastic samples. Thus, it appears that GGT is expressed during the development of malignant squamous epithelial neoplasms of the oral-pharyngeal-laryngeal region. In addition, GGT activity was detected in several samples of nondysplastic epithelium obtained from patients at high risk of developing cancer at these sites. This result raises the possibility that the GGT marker is expressed in very early precancerous sites that have not yet developed the histologic hallmarks of premalignancy. Additional data are required to establish whether the expression of GGT in human squamous mucosa is a useful indicator of partial or complete malignant transformation or whether this phenotypic trait is a tobacco-related or aging-related phenomenon that is independent of malignant transformation (207).

It has been recently found that treatment of rat liver epithelial cells in culture with N-methyl-N'-nitro-nitrosoguanidine (MNNG) produces populations of cells with enhanced specific activities of NADH diaphorase, glucose-6-phosphate dehydrogenase, pyruvate kinase and lactate dehydrogenase, and that these changes precede and accompany the induction of tumorigenic cells. Also, the tumorigenic population was characterized by the presence of approximately 9% of cells with histochemically detectable GGT activity. Single cell cloning was used to isolate cell strains containing high or low GGT activity from the phenotypically heterogeneous population of neoplastically transformed cells. Using the clonal subpopulations isolated, the following phenotypic properties were studied: DNA content; growth rate in culture; GGT, NADH diaphorase, pyruvate kinase, glucose-6-phosphate dehydrogenase, lactate dehydrogenase activities; ability to grow in calcium-poor medium; and ability to form colonies in soft agar. The results showed that none of the above phenotypes cosegregated with tumorigenicity and, therefore, could not be considered as a reliable marker for neoplastic transformation in cultured rat liver epithelial cells. The poor correlations, either qualitatively or quantitatively, demonstrated between paratumorigenic phenotypes and tumorigenicity suggested that neoplastic transformation in these cells involves a specific transforming gene locus or loci and that in vitro paratumorigenic phenotypes are merely epiphenomena of neoplastic transformation and progression (76).

Genetics and Mechanisms of Cell Transformation: In the subject area of genetics and mechanisms of cell transformation are studies designed to test the somatic cell mutation hypothesis of cell transformation and to attempt to identify those specific genes which are responsible or have an influence on cell transformation. There is a large body of data demonstrating a high correlation between the mutagenicity and carcinogenicity of various chemicals. This supports the hypothesis that somatic mutations are involved in the process leading to neoplasia. Somatic cell hybridization techniques have been used to study the types of mutations leading to the transformation of BHK cells by chemical carcinogens. The chemically induced transformants examined show the characteristics of a single-step,

recessive mutation. Temperature-sensitive transformants which result primarily from base change mutations rather than frameshift mutations have also been isolated. Similar types of studies are being conducted to determine the number of complementation groups into which the various transformants isolated fall. This will allow a determination of the number of functional alterations which are needed to lead to the expression of transformation. In another study, the genetic mechanisms of lymphoma induction in mice by the dermal application of 3-methylcholanthrene (MCA) are being investigated. The major goal is to test whether resistance to MCA lymphomagenesis is controlled by a single gene and whether this same gene also confers resistance to radiation lymphomagenesis. This involves the use of various strains of mice in mouse genetic studies. Another approach to the above question involves the use of cells from individuals with a hereditary form of cutaneous malignant melanoma. This disease appears to be inherited as an autosomal dominant trait and most of these patients have preneoplastic melanocyte abnormalities, termed dysplastic nevus syndrome, which are correlated with a markedly increased risk of developing malignant melanoma. The biochemical basis for the increased sensitivity of nonmalignant skin fibroblasts from these patients to 4-nitroquinoline-1-oxide, a UV-mimetic carcinogen will be investigated.

The expression of the anchorage-independent phenotype in BHK 21/13 cells, which have been transformed by chemicals, generally behaves as a recessive trait. From several lines of evidence the acquisition of this trait, although not in itself sufficient to confer tumorigenicity, is suggested as being a necessary event in the multistep progression toward malignancy. Although alterations in a single gene appear to be sufficient to induce anchorage independence, it is not yet clear how many different genes need to be altered in order to make a cell anchorage independent. It is also possible that as a cell progresses toward malignancy, the number of loci at which lesions may induce anchorage independence becomes more restricted. These questions were addressed in one study by testing whether one or more than one gene is involved in the generation of the recessive anchorage-independent phenotype in a matched set of independently derived transformed BHK 21/13 cell clones. When chemically induced and spontaneously transformed cells are fused to the nontransformed parent line, transformation is initially suppressed, but reappears after extended growth of the hybrids. Fusions were performed between 18 spontaneously arising or chemically-induced anchorage-independent transformants and one or more of three similar tester lines. Hybrid populations were screened for genetic complementation as indicated by suppression of anchorage independence. Seventeen out of 18 clones tested were clearly shown to fall into a single complementation group. One clone gave conflicting data, showing rare instances of apparent complementation with a single tester line. Tumorigenicity studies on selected hybrids and parental lines indicated that the in vitro trait of anchorage independence is an accurate indicator of in vivo neoplasia for these cells. The above results indicate that anchorage independence arose in these clones as a result of lesions in the same genetic function and suggests that the final step in the progressive changes of carcinogenesis may frequently be restricted to lesions at a single locus (15).

Newer studies on the role of specific genes and gene products in chemically induced cell transformation have been initiated. The newly developed recombinant DNA, gene cloning, and DNA sequencing techniques have been employed in this research which has resulted in a veritable explosion of publications demonstrating the isolation and characterization of genes responsible for the transformation of cells to malignancy. To date, several different transforming genes have been isolated from different human tumor cells and their homology to various viral oncogenes has been established. Recently, it was shown that there is increased

transcription of the cellular homologue of the transforming gene of Harvey sarcoma virus (c-Ha-ras gene) during the early stages of liver regeneration. Several studies aimed at the identification of specific transforming genes or the activation of known, previously identified oncogenes are currently ongoing or have just been initiated. Various chemically induced animal or cell model systems are being utilized which include rat hepatocellular, mouse thymic lymphomas, rat nasal carcinoma, skin carcinoma, mouse bladder carcinoma and in vitro hamster fibroblast and epithelial cell transformation systems. For example, in one study the expression of the Ha-ras oncogene during the course of bladder carcinogenesis induction, using the N-4-(5-nitro-2-furyl)-2-thiazolyl)formamide/Fischer rat and the butyl-4-hydroxybutyl-nitrosamine/C3H mouse bladder carcinogenesis models, will be evaluated. The studies will involve the quantitation of the mRNA and gene, the sequencing of the gene, localization of the gene within cells and also its localization on the human chromosome. One other study involves the use of the mouse two-stage skin carcinogenesis model to determine whether altered expression of specific genes (several oncogenes, murine leukemia virus proviral sequences, and long terminal repeat sequences) coincides with particular stages of carcinogenesis and/or tumor development.

In experimental models of hepatocarcinogenesis, exposure to carcinogens usually induces focal areas of preneoplastic cells which show a variety of morphological, biochemical, and proliferative alterations that distinguish them from normal hepatocytes. These focal lesions are believed to represent an early stage in the multistep process of carcinogenesis and are most frequently detected histochemically by their enhanced gamma-glutamyl transpeptidase (GGT) activity. A procedure has been recently developed for the isolation of GGT-positive cells using an affinity-purified GGT antibody which has allowed a more detailed analysis of these preneoplastic cells. In view of the possible role of cellular proto-oncogenes in the early stages of carcinogenesis in vivo, it was felt to be important to determine whether preneoplastic hepatic cell populations contained elevated levels of mRNAs coding for the c-myc and H-ras proto-oncogenes. For this study female Sprague-Dawley rats were initiated with diethylnitrosamine following a two-thirds partial hepatectomy and then promoted with phenobarbital for 6 or 11 months. Hepatocytes were isolated and separated into GGT-positive and -negative populations for subsequent analysis. The results of this study suggest that cells from GGT-positive foci do not contain elevated expression of c-myc or H-ras and that increases observed in nodules or tumors may represent a secondary alteration in the regulation of these genes during the multistage process of carcinogenesis. Further research is needed to identify the gene or genes that will be accurate indicators of transformation during the very early stages of hepatocarcinogenesis (157).

There is evidence from studies of mouse thymomas that oncogene activation may be carcinogen-specific. However, for most rodent tumor models and human tumors, the possible role of specific carcinogenic etiology in oncogene activation has not been explored. In order to conduct such a comparative study, DNAs from rat nasal and mouse skin carcinomas and fibrosarcomas induced by the alkylating agents, methylmethane sulfonate (MMS), beta-propiolactone (BPL) and dimethylcarbamyl-chloride (DMCC), were tested for their ability to transform NIH 3T3 cells by DNA transfection. The three compounds produce different DNA adduct patterns when reacted with DNA in vitro, but were shown to induce tumors of the same histological types in the rat nasal cavity or mouse skin. Each of eight MMS-induced rat nasal carcinomas and two of five BPL-induced mouse skin tumors were shown to be positive in the transfection assay, while all of four fibrosarcomas and six carcinomas induced by DMCC were shown to be negative. The transformed phenotype

of the positive transfectants was confirmed by their anchorage-independent growth, tumorigenicity in nude mice, and secondary transfection. The transfectants from MMS-induced tumor DNAs were shown to not contain restriction fragments homologous to rat H-, K- or N-ras oncogenes, although exogenous (rat) tumor-derived DNA sequences were detected in transfected genomes by Southern blot analysis. In contrast, a BPL-induced mouse skin tumor showed evidence of containing activated H-ras. These results suggest a specificity among causal chemical carcinogens for activation of transforming genes in experimental tumors. Further study of animal tumors induced by carcinogens with well-defined biochemical reactivities will be required to elucidate the specific relationships between the biochemical and molecular mechanisms of carcinogenesis (69).

Another possible mechanism of cell transformation by chemicals could involve the induction of DNA sequence rearrangements, free radical intermediates, or specific proteases. These changes could result in the altered cell growth and other properties characteristic of transformed cells. In one study the occurrence of DNA sequence rearrangements during hepatocarcinogenesis in rats and whether such rearrangements involve transforming genes is being examined using gene cloning, restriction endonuclease analysis and DNA transfection technologies. In another project the role of DNA recombinational events, free radical intermediates, cell growth modification, patterns of cell differentiation, and the induction of specific proteases is being examined in the mouse embryo C3H 10T1/2 cell line and in a human diploid cell line transformed by chemicals and radiation.

The transformation of human cells by chemicals or by radiation, as measured by the ability of exposed cells to grow anchorage-independently, has been reported by several laboratories. It has been previously observed that chemically transformed human skin fibroblasts have the ability to degrade type IV collagen, indicating a collagenase activity, whereas normal cells in the same system failed to do so. Collagenases, in addition to plasminogen activator, are a group of proteolytic enzymes which have long been associated with various kinds of tumors and malignant cell cultures. These associations prompted an investigation into the potential biochemical differences with respect to collagenase activity in unexposed and x-ray exposed human cells after extensive subculturing in vitro. Cultures exposed to 100 to 600 rad x-ray doses were serially passaged and checked at various times for growth in semi-solid medium. The presence of cells with the ability to grow under anchorage-independent conditions appeared at 24 population doublings; the frequency of such colonies increased with increasing doses of x-rays. When assayed for plasminogen activator levels, the x-ray-treated cultures at various passages showed insignificant differences from levels observed in control cultures. The amounts of type IV collagen-specific collagenase, however, was shown to increase significantly by 32 population doublings in the x-ray-treated cultures compared with control cultures. The results suggest that the production of type IV collagen-specific collagenase could be useful as an in vitro marker for the transformation of human diploid fibroblasts by x-irradiation (120).

It has been previously reported that the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA) enhances radiation-induced transformation, while several protease inhibitors suppress transformation in vitro. Past experiments have indicated that these agents which modify transformation in vitro are effective during the proliferation of "initiated" cells. Additional studies were performed to define further the conditions in which protease inhibitors and TPA can modify radiation transformation in C3H 10T1/2 cells. The results show that: a) the lowest effective dose of various protease inhibitors to suppress transformation in vitro varies over several orders of magnitude; on a molar basis, the inhibitors of

chymotrypsin appear to be the most effective protease inhibitors; b) antipain and the Bowman-Birk (soybean) protease inhibitor have no effect on radiation transformation when present only during irradiation; c) antipain can suppress radiation transformation in vitro when applied to proliferating "initiated" cells as late as 10 days and 13 cell divisions post-irradiation; and d) TPA treatment following a 10-day protease inhibitor exposure of x-irradiated "initiated" cells does not lead to promotion in vitro. These results suggest that protease inhibitor treatment of the initiated cells has irreversibly reverted cells to their original or "uninitiated" condition which existed before irradiation. Although the present data suggest that there are at least three steps involved in radiation-induced transformation in vitro, it is postulated that there may be even more that cannot be presently distinguished. Further experiments will be necessary to complete our understanding of the different steps involved in the induction of transformation in vitro (120).

Another major focus of projects in this subject area are studies designed to test the cell cycle specificity of the induction of cytotoxicity, mutagenesis, and neoplastic transformation by chemical carcinogens. Also, the quantitative relationship between the level, persistence, and species of carcinogen-nucleotide adducts and the concomitant cell transformation frequency are being determined. There is a substantial amount of information supporting the hypothesis of cell cycle specificity of carcinogenesis. It has been shown that in mouse embryo C3H 10T1/2 cells, G₁ and S phase cells are susceptible to cytotoxicity and mutation, while only S phase cells (in synchronized cultures) are susceptible to neoplastic transformation by exposure to alkylating agents. In adult rat liver, the hepatocytes are generally resistant to carcinogenesis by a single exposure to agents capable of inducing cancer in other tissues. Hepatocyte susceptibility to carcinogenesis is increased by certain treatments which stimulate the proliferation of carcinogen-damaged cells. Additional work is in progress to determine more specifically in rat liver the phase of the cell cycle which is most susceptible to the initiation effect of carcinogenic chemicals.

Development of Carcinogenicity Test Systems and Mechanisms of Mutagenesis: The development of carcinogenicity test systems subject area includes projects in which epithelial and fibroblast cell culture systems, specially constructed bacterial strains, erythroid cells, and a ³²P-labeling test are being used to monitor the effects of exposure to known and potential carcinogens. The end points being measured include cell transformation, mutagenesis, or the generation of DNA damage. Most tumor cells have the capability to proliferate in medium containing low calcium concentrations, while normal cells do not. Mouse epidermal cells can be subcultured in the absence of feeder layers in low calcium medium. In the presence of high calcium medium, these cells cease to grow and terminally differentiate. Epidermal cells altered by chemical carcinogens, however, continue to grow in high calcium medium and do not terminally differentiate. This difference in growth response to high calcium is being used to select cells transformed by carcinogens. Work is in progress to isolate and characterize cloned epidermal cell lines for use in a tester system, to identify and resolve the sources of variability in this system and to identify additional markers of transformation of epidermal cells. Two new mouse epidermal cell lines have been recently isolated and characterized as target cells for three chemical carcinogens. The ability to grow these cells at low cell density of about five clonogenic cells per cm² has permitted more precise quantitation of chemical carcinogen-induced changes in epidermal differentiation. The cell lines, designated 291 and 271c, besides their growth response to calcium, retain other normal properties such as keratin immunofluorescence patterns, ability to form cornified envelopes in response to

Ca²⁺, and a lack of tumorigenicity. Both of the cell lines were shown to have high cloning efficiencies of up to 20% and characteristic epidermal morphology. Chromosomal analysis showed that the chromosome number is near tetraploid. Dose response studies indicated an increase in colonies with altered response to Ca²⁺, proportional to the dose of three chemical carcinogens which were tested: (dimethylbenz(a)anthracene, 3-methylcholanthrene, and N-methyl-N'-nitro-N-nitroso-quanidine). The optimized assay protocol, which was developed, has provided a reproducible means of quantitating carcinogen-altered epidermal cells relative to carcinogen dose and of isolating clones for studies of altered differentiation in carcinogenesis and chemotherapy (130).

The bacterial mutagenesis systems currently in use have been much less successful in predicting the carcinogenicity of metal compounds than they have for predicting the activity of organic compounds. In one project a bacterial short-term test system with a genetic endpoint of broad specificity for detecting carcinogenic metal compounds is to be developed. The investigator has developed a microtitre assay using a number of *E. coli* B strains which allow the analysis of toxicity, lambda prophage induction, and mutagenicity of metal compounds simultaneously. The system will be developed further to increase its sensitivity and to broaden the genetic endpoints detectable. These studies will be extended to the Chinese hamster V79 cell system, which has the known capability to phagocytize insoluble metal compounds. This will allow the study of particulate metal compounds which generally do not enter bacteria.

The proposition that gene rearrangements or gene amplification may be more significant in carcinogenesis than classical point mutations is the rationale for a project to construct a mammalian cell line derived from Chinese hamster V79 cells, which will have deletion mutations in the cell's thymidine kinase (TK) genes and a single copy of the herpes simplex virus TK gene. With this system the principal investigator expects to be able to determine whether a given carcinogen or tumor promoter produces TK+ to TK- mutations and if so, whether these involve base pair substitution, frameshift mutations, or gene rearrangements or gene amplification. Gene amplification or gene rearrangements will be determined by using restriction endonuclease and Southern blot analysis.

There is considerable interest in developing methods that will allow investigators to determine whether people have been exposed to harmful levels of chemical carcinogens. Of the laboratories that are developing such methods, the goal of one is to develop a quantitative in vitro assay for measuring the increase in frequency of thioguanine-resistant T lymphocytes in humans exposed to environmental mutagens. Because practical methods to measure frequency of mutations induced in vivo have not been available, indirect measurements, such as determining the frequency of chromosome damage, sister chromatid exchanges or the induction of tumors are often used. These methods have limitations in applicability due to the relatively low number of cells examined in chromosome-chromatid studies and the long latent period required for cancer. The recent isolation of the T-cell growth factor, interleukin 2, now makes it possible to grow human T-cells isolated from peripheral blood. The proposed series of experiments, if successful, may ultimately allow the evaluation of mutagen dose in cases of human exposure.

The goal of one study is to investigate factors associated with susceptibility to mammary cancer and to develop methods to identify environmental chemicals that may induce mammary cancer. Cultured rat mammary cells are being characterized for use in a direct clonal-specific locus mutagenesis assay system or as activating cells in a cell-mediated mutagenesis assay system. A parallel human mammary gland

in vitro system is also being developed. The susceptibility of rats to polycyclic aromatic hydrocarbon (PAH)-induced mammary carcinomas has been shown to be modified by both genetic background and physiological state. The physiological state of pregnancy has been shown to confer significant resistance to PAH-induced mammary tumorigenesis. Variations in carcinogen metabolism have been suggested as a mechanism of altered susceptibility to chemically-induced tumors in both experimental animals and humans. Results obtained in earlier studies suggested that the ability to activate PAHs is probably not the controlling factor in genetically controlled susceptibility to chemically induced breast cancer. The data suggest that susceptibility is inherited as a dominant trait. Both a single locus autosomal model and an x-linked model have been ruled out. The data obtained support the hypothesis that complete susceptibility is controlled by any one of a group of independently segregating genes; any one gene of this group is both necessary and sufficient to induce maximal susceptibility. It is not known if these genes are identical or different. The results of one study designed to identify the role of these genes suggested that the genes controlling inherited susceptibility are expressed in mammary cells. It has thus far been shown that these genes do not control carcinogen metabolism or activation. Other post activation stages of mammary carcinogenesis are currently under investigation in the rat strains used in the above studies (72).

In parallel studies a specific locus mutagenesis assay using primary cultures of human mammary cells has been developed. It was used to measure mutation frequency at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus in response to ionizing radiation. The data presented demonstrate that the frequency of radiation-induced mutations in breast epithelial cells is very similar to that of transformation-resistant human cell types in vivo such as diploid fibroblasts and T-lymphocytes. Thus, there appears to be no correlation between induction of specific locus mutations and increased susceptibility to cancer induction. The data obtained present evidence that suggests that the initial cellular events following irradiation that lead to cancer induction may not involve specific locus mutagenesis (73).

A method known as the ^{32}P -postlabeling technique has recently been developed for detecting the in vivo formation of carcinogen-DNA adducts in which the use of radiolabeled test compounds is not required. The method involves the reaction of DNA with chemicals in vitro or in vivo and the purification and enzymatic digestion of DNA to deoxyribonucleoside 3'-monophosphates. These are converted to ^{32}P -labeled deoxyribonucleoside 3',5'-bisphosphates after incubation with (gamma- ^{32}P) ATP and T4 polynucleotide kinase. The ^{32}P -labeled digests are then fingerprinted by using reversed-phase liquid chromatography and anion-exchange thin-layer chromatography on polyethyleneimine-cellulose followed by detection by autoradiography and quantitation by scintillation counting. The test conditions have been further refined by chromatographically removing normal DNA nucleotides prior to ^{32}P -labeling. This has led to a greatly increased overall sensitivity of adduct detection and more accurate quantitation of adducts. This method has been applied to answer questions about the tissue specificity of carcinogen adduct formation, the degree of persistence of DNA adducts in cells, and to detect adducts in cells of humans exposed to carcinogenic chemicals.

Chemical carcinogenesis in mouse skin has been shown to be a multistage process, characterized by a long latent period between the exposure to a chemical carcinogen and tumor formation. The initiation phase presumably involves the covalent binding of an ultimate carcinogen to DNA. The precise mechanism underlying tumor initiation and its maintenance over indefinite periods of time are not known, but

a round of DNA replication resulting in adduct-induced miscoding appears to be essential for tumor initiation. Although the initiated state induced in mouse skin by 7,12-dimethylbenz(a)anthracene (DMBA) has been shown to persist virtually indefinitely, DMBA-DNA adducts in mouse skin have been analyzed, until recently, only for a period of 8 days posttreatment. The ^{32}P -postlabeling assay has been used to investigate, in detail, the persistence of DMBA-DNA adducts in mouse skin for a much longer time than previously possible. Following a single dose of DMBA, DNA was isolated from epidermis and dermis at several time points from 24 h to 42 weeks later. Total DNA binding at 24 h was determined in epidermal and dermal DNA to be about 34 and 28 adducts per 10^7 normal nucleotides, respectively. Although initial binding was higher in epidermal DNA, the adducts were shown to be about 10 times more persistent in dermal DNA. At 42 weeks, total binding levels were shown to be about 0.17 and 1.7 adducts per 10^7 nucleotides in epidermal and dermal DNA, respectively. From ^3H -thymidine labeling experiments at 40 weeks post DMBA treatment, no significant difference of label incorporation into DMBA-modified and control DNA was found. Thus, it was concluded that the bulk of the persistent adducts was present in subpopulations of dormant cells. These cells are hypothesized to be incapable of division due to the adduction and/or mutation of genes critical for growth and may thus correspond to "latent tumor cells." The observations made in this study suggest a possible relationship between adduct persistence and initiation persistence (177).

In another study the question of DNA adduct formation in diethylstilbestrol (DES)-induced renal carcinogenesis in vivo was investigated. There is a current controversy concerning the mechanism of carcinogenesis by DES. DES and other synthetic and natural hormones have been shown to act as promoters of tumorigenesis and have thus been viewed as examples of epigenetic carcinogens, compounds that can induce cancer without direct interaction with genetic material. This view is supported by the failure of DES or its derivatives to cause mutations in the Ames assay. However, DES has been shown to induce sister chromatid exchanges, unscheduled DNA synthesis and aneuploidy in Syrian hamster embryo cells, suggesting that DES does cause damage to cellular macromolecules. An attempt to search for covalent DES-DNA adducts using ^{14}C -labeled DES resulted in the observation of extremely low levels of binding in rats and hamsters and unsuccessful attempts to chromatograph and isolate adducts. From these results it was concluded that the covalent modification of DNA by DES is not relevant to carcinogenesis by DES. In the present study the ^{32}P -postlabeling assay detected the presence of covalently modified DNA nucleotides in the kidneys after chronic DES treatment, but not in kidneys of untreated hamsters or in liver or tumor tissue of DES-treated animals. At least three different adduct spots were detected. Quantitative estimation by scintillation counting indicated that, at the 5 month time point, one nucleotide in $1.5\text{--}2.0 \times 10^7$ nucleotides was modified. This is the first demonstration of the ability of an estrogen to give rise to covalent DNA modification in vivo specifically in the target organ of carcinogenesis. The structures of the modified nucleotides have not yet been determined. Recent observations, however, show that the DNA adducts observed do not contain estrogen-derived moieties. The above results indicate that DES can be classified as a complete carcinogen and has led to the conclusion that estrogens such as DES induce the binding of some endogenous compound(s) to target tissue DNA (177).

In another application of the ^{32}P -postlabeling method, the presence of covalent DNA adducts in human full-term placental samples was investigated. Maternal blood and cord blood samples and placentas were obtained at delivery from volunteers. Blood specimens were analyzed for three biochemical markers of smoking exposure--cotinine, thiocyanate, and carboxyhemoglobin--and smoking data was collected from

each volunteer. DNA was also analyzed for adducts by a competitive enzyme-linked immunosorbant assay (ELISA) with antibodies to DNA modified by anti-benzo(a)pyrene diol epoxide (BPDE-I). The ELISA detected a small but not statistically significant increase in adduct levels in placental specimens of smokers. The post-labeling assay detected up to five modified nucleotides, one of which (adduct 1) was strongly related to maternal smoking during pregnancy. This adduct was shown to be present in placental tissue from 16 of 17 smokers, but in only 3 of 14 non-smokers. Adduct 1 was presumed to be a derivative of an aromatic carcinogen, but when cochromatographed with test DNA preparations containing DNA adducts from aromatic carcinogens known to be present in cigarette smoke (benzo(a)pyrene, benz(a)anthracene, dibenz(a,h)anthracene, pyrene, chrysene, fluoranthene, benzo-(g,h,i)perylene, 4-aminobiphenyl, 2-naphthylamine or the isomeric methyl or ethyl derivatives of aniline), none cochromatographed. Efforts are continuing to identify this adduct. The data show the association of cigarette smoking with covalent damage to human DNA in vivo and demonstrates that this analytical approach should contribute to a definition of chemical components of cigarette smoke as well as other environmental exposures that most severely damage human DNA (177).

In addition to the development of mutagenicity test systems, projects are being supported which seek to understand how mutations and DNA or chromosome damage are generated by carcinogenic chemicals. Specifically synthesized oligonucleotides of defined base sequence are being used to examine the molecular mechanism of frameshift mutagenesis. The base sequence specificities of the interactions of frameshift mutagens with oligonucleotides are being studied and correlated to their mutagenic activity in Ames tester strains having known base composition in the frameshift site. Studies are also being supported which seek to understand the mechanism and genetic control of frameshift mutagenesis in yeast. The recently sequenced His4 gene system with existing (+1 G/C) and new (-1) frameshifts are to be used to construct tester strains. DNA sequencing and recombinant DNA technology have been used in these studies. The types of studies to be undertaken include an assessment of the effect of transcription on mutation frequency, the effect of having an origin of replication in close proximity, an analysis of gene duplication, and a study of the potential role of the nuclear envelope in mutagenesis.

Newer studies in this area have focused on the use of specific genes which will either be cloned into plasmids or are present in cellular DNA as targets for the mutagenic action of various chemical carcinogens. The c-Ha-ras oncogene cloned into a plasmid, the lac gene introduced into M13 phage DNA, and the dihydrofolate reductase gene in Chinese hamster ovary cells are being used as target genes to assess the mutagenic action of chemicals such as benzo(a)pyrene diol epoxide, N-acetoxy-2-acetylaminofluorene, and other aromatic amines. DNA sequence techniques and effects of lesions on DNA synthesis will be used to determine the mechanisms of mutagenesis.

In three other studies, methods for the analysis of mutations induced in human or other mammalian cells at the DNA sequence level are being developed. The approaches used depend on the development of recombinant DNA shuttle vectors composed of the simian virus (SV40) early region, the Epstein-Barr virus (EBV) oriP element or some other sequence that allows the plasmid to be replicated in human or mammalian cells, sequences derived from the bacterial plasmid pBR322 which permits the plasmid to also replicate in E. coli, and some selectable target gene for mutant selection. The studies seek to determine the types of DNA sequence changes induced by chemical carcinogens or mutagens and to characterize

host processes that determine the frequency or types of mutations induced specifically in mammalian cells.

A stumbling block to the use of available shuttle vectors for the study of mutagenesis has been the observation that the process of transfection of DNA into mammalian cells is, itself, highly mutagenic. Plasmid DNA isolated from a variety of mammalian cells shortly after transfection of papovavirus-based shuttle vectors was found to contain a high frequency of mutations (about 1%). In another report, a recombinant shuttle vector containing the entire bovine papillomavirus (BPV) genome, sequences from pBR322 and the *E. coli* *gpt* gene was observed to have a spontaneous mutation frequency of $3 \text{ to } 16 \times 10^{-5}$ following passage through mouse C127 cells. Restriction digest analysis indicated that most of the mutant plasmids had gross rearrangements in their DNA structures (40).

One exception is the shuttle vector system developed by Dr. M. Calos (26). It is an SV40-based shuttle vector with the *lac I* gene as the target gene for mutagenesis. It was found to replicate well in the human cell line 293 with a relatively low spontaneous mutation frequency. In one study the mutations induced by UV light in this system were analyzed. The nucleotide sequence changes for 53 UV light-induced mutations, as well as 32 spontaneous point mutations, were deduced by genetic techniques which are possible with this system. Most of the mutations were shown to be transitions (G-C to A-T) and to occur at pyrimidine-pyrimidine sequences. The observed mutagenic specificity closely resembled that of UV light in *E. coli*, suggesting that human and bacterial cells respond similarly to damage from UV light. These results demonstrate that the use of the *lac I* shuttle vector in this way should permit the determination of the mutagenic specificity of a wide range of mutagens and carcinogens in human cells.

In another laboratory, a recombinant DNA shuttle vector was developed which is able to replicate as a plasmid in *E. coli* and in EBV-transformed human lymphoblastoid cell lines. It contains the herpes simplex virus type I thymidine kinase gene (HSV *tk*) as the target for mutagenesis studies. The vector is introduced into the EBV-transformed lymphoblastoid cell line (LCL-721) by electroporation. About 2% of the transfected cells expressed the vector-encoded gene for hygromycin resistance which is used to select those cells containing the plasmid. The spontaneous mutation frequency of the HSV *tk* gene was determined to be 6×10^{-5} . Treatment of plasmid-bearing LCL-721 cells with N-ethyl-N-nitrosourea resulted in a dose-dependent increase of up to 15-fold in the frequency of mutations in the HSV *tk* gene. The dose-response for the induction of mutations in the plasmid-encoded gene was shown to closely parallel that for the induction of mutations in the cellular gene for hypoxanthine (guanine) phosphoribosyl transferase. A small fraction (30%) of the induced mutations were shown to be deletions. This is in contrast to the large fraction (85%) of the transfection-induced mutations which were shown to be deletions (47).

Properties and Mechanisms of Tumor Promotion: Research in this subject area involves projects designed to analyze the various cellular, biochemical and molecular activities and pleiotropic effects induced in cells upon exposure to tumor promoters. The phorbol ester tumor promoters are, by far, the most widely used compounds in these studies. They have been shown to exert their effects by binding to specific receptors on cell surface membranes. A number of grants support studies on the characterization of the phorbol ester receptor protein. The results of phorbol ester binding include alterations in membrane phospholipid metabolism, membrane structure and function, alterations in the transport of small molecules, the activation of macromolecular synthesis, the induction or inhibition

of terminal cell differentiation by normal or neoplastic cells and the mimicry of the transformed phenotype by normal cells and the enhancement of transformation by chemicals and oncogenic viruses. Two of the studies are focused on the perturbation of ion fluxes by the tumor promoter, 12-O-tetradecanoyl-phorbol-13-acetate (TPA). Since the action of TPA may be mediated by the phosphorylation of proteins and lipids, several studies are focused on the purification and characterization of protein kinase C, a calcium and phospholipid-dependent protein kinase whose activity is stimulated by TPA. A characterization of the proteins phosphorylated by this enzyme is included in some of these studies. The role of free radicals in promotion, either the active oxygen species generated by TPA in cells or the hydroperoxy fatty acids generated during the induction of the arachadonic acid cascade by TPA and other first and second stage promoters, is the focus of several of the studies. The activation of expression of certain genes is thought to occur during neoplastic progression of cells. The possible activation of oncogene sequences and other viral and cellular gene sequences by TPA and other promoters is the focus of at least five studies. Another property of tumor promoters is their apparent ability to disrupt cell-cell communication. The mechanism of this phenomenon is the focus of at least three studies and systems exhibiting this phenomenon are being developed as indicators of potential tumor promoting agents.

Since humans are not normally exposed to phorbol ester tumor promoters, it was deemed necessary, in 1981, to stimulate more research on agents more relevant to human exposure which might function as tumor promoters. To accomplish this a Request for Applications (RFA) was issued inviting grant applications from interested investigators for both basic and applied studies that would seek to provide insight and approaches to an understanding of the role of tumor promoters, hormones and other cofactors in human cancer causation. The studies were to be focused on one or more of five different categories: (1) the development of nonphorbol tumor promotion or cocarcinogenesis models in experimental animals using the breast, colon, lung, prostate, stomach, urinary bladder, and/or uterus organ systems; (2) the development of nonphorbol tumor promotion or cocarcinogenesis models in human and/or nonhuman cell and/or organ culture systems; (3) the study of the possible tumor promotion role of hormones and substances such as bile acids, saturated/unsaturated dietary fat, alcohol, salt or oxygen-free radicals; (4) the identification and elucidation of the mechanisms of action of nonphorbol tumor promoters and/or cocarcinogens; and (5) interdisciplinary studies involving epidemiologists and experimentalists to test hypotheses concerning tumor promotion generated by either.

In FY82 12 grants were funded from applications submitted in response to this RFA; ten were approved for 3 years of funding and two for 4 years. The role of dietary fat on DMBA-induced mammary carcinogenesis in rats or mice was the focus of two of the studies. The cocarcinogenic action of ethanol with nitrosamines in the oral cavity, esophagus and larynx of rats, mice and hamsters was the focus of one study. The rates of metabolic activation of nitrosamines in the target organ and cell cultures was to be measured. In a mouse lung tumor model, the mode of action of butylated hydroxytoluene (BHT) as a tumor promoter was to be examined. The metabolism of BHT, the activation of cyclic GMP- and calcium-dependent protein kinase, the effect of glucocorticoids on urethane tumorigenesis and tumor promotion and the effect of BHT on glucocorticoid receptor localization was to be studied. Using a heterotopically transplanted rat bladder system, one laboratory was to investigate the promoting effect of urine components on bladder carcinogenesis induced by N-methyl-N-nitrosourea and N-butyl-N-(3-carboxypropyl)-nitrosamine. The hypothesis that asbestos and selected nonasbestos minerals act as tumor promoters in carcinogenesis of the respiratory tract was to be studied using

a hamster trachea model. The determination of whether EBV-related oncogenic mechanisms in in vitro virus-cell interaction models involves promotion was to be made in one laboratory. The hypothesis given is that a viral-mediated increase in an intracellular protein that blocks the viral lytic cycle and interferes with cell differentiation leads to uncontrolled cell proliferation and the ultimate selection of neoplastic cells. A study of the tumor-promoting activity of a number of anthracene derivatives such as chrysarobin and its synthetic analogs and homologs, which are related to anthralin, was to be conducted using the 7,12-dimethylbenz(a)anthracene skin tumor model system. Two in vitro model systems for testing for tumor promoters were to be developed. One model system used hepatocytes or liver cells from carcinogen-treated rats which were then promoted in culture using selected compounds. The other model system used various rodent and human cells to test the hypothesis that the induction of mutations at the HGPRT locus by promoters in hypermutable cells is a common property of cancer cells. In one study on the mechanism of action of promoters, the ability of promoters to stimulate gene amplification to methotrexate resistance was to be studied. Finally, one of the studies involved a biochemical epidemiology project in which sex hormone levels in breast and prostatic cancer was studied.

In FY85 two of the projects, successfully recompleted, were renewed and funded. In FY86 two and possibly three additional projects will be renewed and funded by the end of the fiscal year, mainly due to successful revised renewal applications. Three of the projects will not be renewed as no renewal applications have been submitted and none are anticipated. One renewed project is for studies on the mechanism of action of a nonphorbol ester tumor promoter, chrysarobin. The second project funded in FY85 supports studies on the potential role of oxygen-free radicals in asbestos-induced bronchogenic carcinoma. Of the projects renewed in FY86, one involves the further investigation of mechanisms of urinary bladder carcinogenesis utilizing the heterotopically transplanted bladder system and natural bladder models in rats. In a second project the goal has been changed to investigate the possible role played by the endogenous mouse mammary tumor virus gene and cellular oncogene components in murine mammary tumorigenesis, alone and in combination with chemical carcinogens. The third project involves studies on the role of Epstein-Barr virus in neoplastic transformation. It is also evident that this RFA has stimulated more studies on nonphorbol tumor promoters of relevance to humans. Some recently funded projects seek to study the activity of compounds such as n-alkanes, cyclosporin A, endogenous hepatic growth modulators, hormones and dietary L-tryptophan as tumor promoters. In addition, epithelial cell and organ culture systems from human endometrium and human prostate are being developed to study the process of tumor promotion by a variety of agents such as hormones and TPA.

Tumor promoting phorbol esters have been shown to affect cellular growth and differentiation as well as to interact specifically with a cellular receptor protein. The phorbol ester receptor has been shown to be tightly associated with a calcium-activated, phospholipid-dependent protein kinase (PK-C). PK-C can be activated by calcium in the presence of phospholipid or by phorbol esters or diacylglycerol in the presence of suboptimal amounts of calcium. The phorbol ester receptor and PK-C activity are present in both the soluble and particulate fractions of cells and tissues. Studies were done to clarify the relationship between the activation of PK-C, phorbol ester binding to the receptor and induced alterations in the subcellular distribution of the complex. The data obtained showed that phorbol esters caused the phorbol ester receptor in the soluble fraction of isolated mouse spleen lymphocytes to become associated with the particulate fraction. This effect paralleled the shift of PK-C activity to the

membrane fraction induced by phorbol esters. The data are consistent with the interpretation that a single protein possesses both activities. The observations made support the hypothesis that the cell membrane is the locus of action of both the phorbol esters and the PK-C activity (5).

Phorbol esters have been shown to mediate reversible down-regulation (i.e., internalization) of the surface transferrin receptor. Down regulation was shown to be tightly coupled with increased receptor phosphorylation, while up-regulation is associated with receptor dephosphorylation. Increased phosphorylation of the receptor has been shown to be mediated directly by activated protein kinase C (PK-C). A primary product of phosphatidylinositol metabolism, 1,2-diacylglycerol, has been found to bind similarly to PK-C as the phorbol ester tumor promoters and activate PK-C. Using transferrin receptor-containing HL-60 cells, the hypothesis that *sn*-1,2-diacylglycerols function as intracellular activators of PK-C in intact cells and whether such a response is spontaneously reversible was tested. The effect of the synthetic diacylglycerol, *sn*-1,2-dioctanoylglycerol (*diC₈*), on the expression of the surface transferrin receptor demonstrated that exogenous *diC₈* was able to act as an intracellular activator of PK-C and to stimulate both down-regulation and increased receptor phosphorylation in a manner similar to that induced by the active tumor promoter, 4 beta-phorbol-12,13-dibutyrate (PDBu). When PDBu was added, the effect noted was spontaneously irreversible. The same effect mediated by *diC₈* was shown to be brief, lasting only minutes, and to be spontaneously reversible. The rate of reversibility was shown to be dependent on the concentration of *diC₈* added and was associated with rapid formation of a newly detected intracellular phospholipid that corresponded to *sn*-1,2-dioctanoyl phosphatidic acid. These results, in conjunction with findings that demonstrate that exogenous diacylglycerols (including *diC₈*), when added to cells, do not stimulate cellular phospholipase A₂ or C, argue that PK-C is activated only briefly in this system since exogenous *diC₈* is subject to rapid intracellular metabolism to phosphatidic acid. Such rapid reversibility would represent a convenient method of intracellular regulation of this important enzyme. The sustained stimulation of PK-C by any nonmetabolized or slowly metabolized agent, such as phorbol esters, may account for their observed effects on growth control such as tumor promotion and induction of cellular differentiation and/or proliferation (152).

The mouse genome contains a retrovirus-like sequence, designated VL30, which is expressed at high levels in transformed cells and which can be induced by exogenously supplied epidermal growth factor (EGF). Although VL30 transcripts corresponding to at least one open reading frame within the VL30 long terminal repeat region are easily identified, it is not yet known whether transcription of VL30 results in the appearance of a cellular polypeptide. However, because VL30 elements are responsive to EGF and in view of the known enhancer activity of retroviral repeats, VL30 transcription is proposed as a model system for investigating the regulation of specific gene transcription by polypeptide growth factors. The binding of EGF to the EGF receptor has been shown to produce changes in intracellular calcium levels and in phospholipase activity which indirectly lead to activation of protein kinase C (PK-C). Various cells, including an EGF receptorless variant and a phorbol ester nonresponsive variant, were treated with various phorbol ester tumor promoters and with the synthetic diacylglycerol, *sn*-1,2-dioctanoylglycerol (*diC₈*) in one study. Tumor promoting phorbol esters were shown to increase the level of VL30 expression. Stimulation with the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA) or EGF produced a similar time course of VL30 expression. TPA was shown to induce VL30 expression in the EGF-receptorless NR6 cell line, indicating that neither EGF ligand-receptor binding nor phosphorylation of the EGF receptor was required for induction of VL30

expression. Protein synthesis was shown not to be required for the TPA-mediated increase in VL30 expression, which was also shown to be stimulated by treatment with diC_8 , an analog of a probable endogenous activator of PK-C. These results suggested that PK-C plays a direct role in regulating VL30 gene expression. The identification of the substrates used by PK-C in the induction of VL30 expression may contribute to the understanding of the ways in which protein kinases can regulate the activity of specific genes (144).

A substantial amount of evidence has been accumulating in the last few years that suggests that the generation of free radicals, such as superoxide anion and hydroxyl radical, may be involved in the tumor promotion stage of multistage skin carcinogenesis. The most direct evidence for free radical involvement comes from studies in which free radical generating compounds, such as benzoyl peroxide, were shown to be complete tumor promoters. Indirect evidence comes from studies in which various antioxidants were shown to be inhibitors of TPA-induced tumor promotion. TPA has also been shown to cause a substantial decrease in the basal level of superoxide dismutase (SOD) and catalase, the major detoxification enzymes for superoxide and H_2O_2 , respectively. The stimulation of oxygen-free radicals by TPA has been measured using a chemiluminescence assay and found to be inhibitable by SOD and retinoids. Since many tumor promoter effects may be related, directly or indirectly, to free radicals, it was deemed to be of interest to determine the cellular source of these radicals. For these studies a chemiluminescence assay was developed for mouse epidermal cells and the chemiluminescence response was measured and shown to be TPA dose dependent, cell number dependent, and largely inhibitable by SOD. Catalase, which is specific for H_2O_2 , and mannitol, which is a scavenger for hydroxyl radicals, had negligible inhibitory effects. Inhibitors of various parts of the arachidonic acid cascade were found to affect the TPA-induced chemiluminescence response in a manner that corresponded to their effects in *in vivo* tumor promotion experiments. Agents which are predominantly lipoxygenase inhibitors or agents which are effective against both lipoxygenase or cyclooxygenase were shown to be effective in diminishing the chemiluminescence response. Cyclooxygenase inhibitors were shown to have no, or a slight enhancing, effect at low doses. The data obtained suggest that at least a major part of the TPA-induced chemiluminescence response is due to the metabolism of arachidonic acid, most probably by the lipoxygenase(s). This conclusion was supported by a further study in which a chemiluminescence response was generated by the treatment of mouse epidermal cells with phospholipase C. The specificity of the response to phospholipase C from *C. perfringens* and not from *B. cereus* or phospholipase A_2 suggested that specific phospholipids are involved. The response observed is interpreted to arise from the phospholipid-protein kinase C model for phorbol ester binding and activity (61).

In one of the studies formerly supported under the "Promotion" RFA, the mechanism of action of asbestos-induced bronchogenic carcinoma is being investigated. The cellular mechanisms of injury by fibers is being actively pursued by many laboratories in an effort to understand the pathogenesis of asbestos-related diseases. Experiments have been complicated by the fact that asbestos is not one, but a family of minerals, each with individual physicochemical features. Cytotoxicity by chrysotile and crocidolite (the more extensively studied fibers) is attributed to various features of these minerals including fibrous geometry, size, surface area and charge. Experiments with chrysotile have suggested that interaction of positively charged fibers with membranes is intrinsic to cell injury. Since oxygen-free radicals are generated from membranes in response to a number of perturbations, the hypothesis that asbestos-induced injury might involve the production of free radicals was tested. Asbestos-induced cytotoxicity in a

hamster tracheal epithelial cell line was shown to be inhibited by superoxide dismutase (SOD), a scavenger of superoxide, and by mannitol and dimethylthiourea, scavengers of the hydroxyl radical, when cells were exposed to long (greater than 10 micrometers in length) fibers of chrysotile and crocidolite. In contrast, injury to epithelial cells by short (less than 2 micrometers) chrysotile or glass fibers was not prevented by scavengers of superoxide, hydroxyl radical, H_2O_2 or singlet oxygen. These results implicate active oxygen species as mediators of injury by long asbestos fibers to cells of the respiratory tract. Appreciable amounts of copper-zinc SOD was detected in hamster tracheobronchial epithelial cells and alveolar macrophages in vitro and in histologic sections of rat and human respiratory tract using immunochemical and biochemical techniques. The activity of total endogenous SOD (copper-zinc and manganese forms) was shown to increase in tracheal epithelial cells exposed for several days in vitro to either crocidolite or chrysotile asbestos, but was unchanged in untreated cells and in those exposed to comparable levels of glass fibers. Following inhalation of asbestos by rats or exposure of cells in culture to asbestos, long fibers were observed protruding from both epithelial cells and alveolar macrophages. The unsuccessful phagocytosis of long fibers of asbestos, coupled with the generation of oxygen-free radicals, is thought to be responsible for the pathogenic potential of long fibers in asbestos-associated diseases of the respiratory tract (164).

Interspecies Comparisons in Carcinogenesis: In the subject area of interspecies comparisons in carcinogenesis are studies undertaken as a result of a specific initiative from the Branch to fill a perceived need to develop scientifically sound methodology for the extrapolation of carcinogenesis data derived from studies on experimental animals to humans. The initiative was designed to encourage studies that would be supportive of the Environmental Protection Agency in the area of risk assessment. In 1980, an RFA was issued inviting grant applications from interested investigators for both basic and applied studies designed to provide insight and approaches to an understanding of similarities and differences in the response to chemical carcinogens between experimental animals and humans. The proposed studies were to emphasize the use of accessible human cells, tissues, body fluids and excreta and to focus on quantitative relationships related to the carcinogenesis process.

In FY 81, 16 grants were funded from applications submitted in response to this RFA; 15 were approved for 3 years and one for a 5 year period. Fourteen of the grants supported studies with either human cells only or with human and other rodent or monkey cells. One grant supported comparative studies in mice and rats only and another used hamsters, mice, and rats. Fourteen of the grants supported studies on the comparative metabolism of a variety of chemical carcinogens such as polycyclic aromatic hydrocarbons, aromatic amines, and nitrosamines. The development of human hepatocyte, pancreas, esophagus, and bladder in vitro cell transformation systems was the additional goal in four of the projects. The development of direct or cell-mediated mutagenesis or genetic damage assay systems was also the focus in three of the studies. The primary goal in two of the funded studies was to develop techniques to measure mutant proteins in peripheral blood lymphocytes or in red blood cells from individuals who had been exposed to potential mutagens or carcinogens either as the result of various clinical procedures or from environmental or occupational exposure to chemicals.

By the end of FY86, 6 of the 16 original projects will still be active. Five investigators have chosen not to submit competing renewal applications. Of the 11 applications for which competing renewal applications were received, three were renewed in FY84, one in FY85, and two in FY86. The main goal of one of these

projects is to provide an understanding of the mechanisms responsible for the metabolic activation, organ specificity, and species and strain specificity of the carcinogen, methylazoxymethanol. Another project involves the continued development of equivalent rat and human mammary gland in vitro systems for ultimate interspecies comparisons of interactions of chemical carcinogens with these cells. The third project involves the further development and characterization of cell culture and transplantation systems of human hepatocytes obtained from normal and cirrhotic liver. A study of the mechanism of pancreatic carcinogenesis by N-nitrosomethyl(2-oxo-propyl)amine and related compounds in rats and hamsters is the goal of the fourth project. The fifth project involves studies on the perfection, characterization, and application of a 6-thioguanine resistance assay in human peripheral blood cells for monitoring environmental mutagens. The final active project supports a continuation of studies on the characterization of cytochrome P-450 isozymes in humans in which biochemical, immunological, and molecular biological techniques are used to define the molecular basis for the genetic polymorphism of certain oxidative activities in humans. Only one of the six renewal projects is still included in this subject area classification. The other projects are included in other subject areas which are now more appropriate.

While it is not yet clear what the overall impact of the funded studies will have on the ability to extrapolate animal carcinogenicity data to humans, it is clear that the initiative on interspecies comparisons in carcinogenesis has stimulated additional studies using cells from human tissues, which will increase our knowledge on the metabolism and processing of carcinogens by those tissues and on the biological and molecular characteristics of cells transformed by chemical carcinogens. In addition, many more investigators now utilize more than one species or strain of animals in their proposed studies. Different animal species have been shown to be either sensitive or resistant to the action of various xenobiotics. Thus, use of different species can give insights into the mechanism of action of carcinogens in carcinogenesis. Subsequent to the RFA, three other projects have been added to this category of research.

It had been previously established that there are interindividual variations in humans in the metabolism of foreign compounds, such as therapeutic drugs. Genetic polymorphisms contribute in large part to the large interindividual differences observed. Debrisoquine 4-hydroxylase activity is considered to be a prototype for genetic polymorphism in oxidative drug metabolism in humans; about 10% of Caucasian populations exhibit a poor metabolizer phenotype, and the clearance of at least 14 other drugs has been shown to be deficient in patients exhibiting this phenotype. The formation of 4-hydroxydebrisoquine, a major urinary metabolite in normal individuals, is apparently under the control of a single autosomal genetic locus. The deficiency of debrisoquine hydroxylation is an autosomal recessive trait. Prior to conducting studies on the human debrisoquine 4-hydroxylase enzyme, a minor cytochrome P-450 was purified (to homogeneity) from Sprague-Dawley rat liver using debrisoquine 4-hydroxylase activity as an assay. This cytochrome P-450 (designated P-450_{UT-H}) was characterized by gel electrophoresis, peptide mapping, and immunochemical analysis. Antibodies prepared to the rat cytochrome P-450_{UT-H} were used in subsequent immunocharacterization experiments. Techniques developed for the purification of the rat P-450 were modified to isolate a microsomal P-450 (designated P-450 DB) from several different human livers. These showed high catalytic activity towards debrisoquine metabolism (sparteine, bufuralol, encainide, and propranolol). Another human liver P-450 was also purified (designated P-450 PA) which possessed relatively high phenacetin O-demethylase activity and may be involved in the variation of this activity among

humans. Polyclonal antibodies were raised to the two enzymes. The two enzymes and their activities were characterized and distinguished by chromatographic separation, SDS-polyacrylamide gel electrophoresis, amino acid composition, immunoinhibition studies, and steady-state kinetic assays. The immunochemical studies suggested that each form represents only a small fraction of the total cytochrome P-450 in human liver microsomes. These biochemical studies provide a basis for better understanding the mechanisms which underlie genetic polymorphisms involving cytochrome P-450 in humans (82).

The development of animal models for the study of pancreatic cancer is an important goal. One of the most successful is the hamster, in which pancreatic ductal carcinomas are induced in the pancreas by N-nitroso-2,6-dimethylmorpholine (NNDM), N-nitrosobis(2-oxopropyl)amine (BOP) and other N-nitrosamines. This model has received additional attention since both BOP and NNDM fail to induce pancreatic cancer in the rat. The different effects of the two nitrosamines have been attributed to metabolic differences in the two species; however, in vivo studies have revealed more similarities than differences. A common metabolite of BOP and NNDM, N-nitroso(2-hydroxypropyl)(2-oxopropyl)amine (HPOP), has been found to induce pancreatic tumors in both the hamster and rat. A similar effect occurs with N-nitrosobis(2-hydroxypropyl)amine (BHP), which is a secondary metabolite of NNDM and BOP formed by the reduction of HPOP. In vitro metabolism studies have shown that rat liver or pancreas microsomes are about sevenfold less active in metabolizing NNDM or BOP. In this study rats and hamsters were administered a single dose of HPOP and the urinary metabolites were examined at various time intervals. The data obtained suggested that hamsters reduce HPOP to BHP more efficiently than rats, while rats are more effective in forming their glucuronide conjugates. Hamsters were shown to differ significantly from rats in their capacity to form and excrete the sulfate ester of HPOP. The absence of sulfate esters of HPOP in the rat is thought to be attributed to either the preferential glucuronidation of HPOP or the lack of an appropriate sulfotransferase. In further experiments to study the species differences in response to nitrosamine carcinogenesis, a pancreatic acinar cell-mediated mutagenesis assay was developed to study the metabolism of BOP and HPOP to forms mutagenic for Chinese hamster V79 cells. Acinar cells of both species were shown to be capable of activating BOP and HPOP to mutagens for V79 cells in a dose-dependent manner. BOP was shown to be a considerably more potent mutagen than HPOP after activation by either cell type. This is consistent with the known in vivo specificity of BOP versus HPOP in the hamster pancreas. The comparable abilities of rat and hamster acinar cells to convert BOP or HPOP to mutagenic forms imply that pancreatic metabolic activation alone cannot explain the difference in organotropism of BOP and HPOP in the two species (194).

Genetics and Regulation of Enzymes Associated with Carcinogenesis: Research projects in this subject area are focused on the use of somatic cell genetic and molecular biological approaches to study the regulation of the levels of carcinogen metabolizing enzymes. In nine of the projects, the development and use of cloned cDNA probes to cytochrome P-450 or epoxide hydrolase genes form part of the proposed studies. These cloned cDNA probes are used to examine the levels of expression of the mRNAs for the genes in response to the modulation of enzyme activity by various chemicals. The genomic organization of the genes can also be examined. In two of the projects, mutants or variant cells, altered in their ability to induce the carcinogen metabolizing enzymes, are being isolated and characterized. Using the inbred hamster model the genetic variation in aryl-hydroxamic acid acyltransferase, sulfotransferase, N-acetyltransferase, and N-deacetylase enzymes which are involved in the metabolism of aromatic amines is

being studied. The characterization, localization and regulation of the enzyme, arylsulfotransferase IV, is the focus of one other study. Also, the isolation and characterization of carcinogen-binding or receptor proteins, which may play a role in the regulation of cytochrome P-450 gene expression, is being pursued in two of the studies.

The cytochrome P-450 monooxygenase system is a large family of enzymes responsible for the oxidation of drugs and environmental pollutants, as well as many endogenous compounds. Results from enzyme purification studies and cloning of the respective mRNAs indicate that many of these enzymes are encoded by distinct structural genes and that they are under different modes of regulation. Treatment of rabbits with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) results in the induction of two major forms of cytochrome P-450 in liver, P-450 4, and P-450 6. These two P-450s have been shown to be under differential regulation with respect to age and tissue specificity. Cytochrome P-450 4 was shown to be the major adult form which appears to be present only in liver, whereas P-450 6 was shown to be inducible in most tissues and is present in neonates as well as adults. Recently, cDNAs encoding TCDD-inducible rabbit P-450 4 and P-450 6 have been isolated and characterized by initially using monoclonal antibodies to P-450 6 to identify several recombinant clones from a pBR322 cDNA library. DNA sequence analysis demonstrated that the P-450 6 mRNA is similar in homology to rat P-450c and mouse P₁-450, while the P-450 4 mRNA is more homologous to rat P-450 d and mouse P₃-450. Blot hybridization analysis demonstrated that TCDD dramatically induced P-450 4 and 6 mRNAs, which differ in size (about 2,000 and 2,300 bp, respectively). The sizes of these mRNAs was shown to differ from their analogs in the mouse as a result of a difference of greater than 300 bp in the 3' untranslated portions of the mRNAs. In addition, two different human cDNAs that cross-hybridize to TCDD-inducible P-450 4 and P-450 6 cDNAs have been isolated and characterized. DNA sequence analysis demonstrated that the human P-450 4 cDNA clone is 83% and 75% homologous to rabbit P-450 4 and P-450 6, respectively. The human P-450 6 cDNA clone was shown to be 79% and 72% homologous to rabbit P-450 6 and P-450 4, respectively. A comparison of the DNA sequence of the two human cDNA clones shows that they are about 80% homologous, a result which is similar to the degree of homology found between rabbit P-450 4 and 6 cDNA sequences. Human P-450 4 mRNA has been shown to be about 3,000 bases, while human P-450 6 mRNA is about 2,600 bases in length. The data show that both human P-450 4 and 6 cDNAs recognize different genomic fragments, indicating that each is encoded by different genes. These results indicate the presence of at least two P-450 genes in humans that are highly homologous to the TCDD-inducible P-450s in rabbits and mice (219).

The treatment of animals with any of a variety of polycyclic aromatic hydrocarbons (PAHs) results in the coordinate induction of several drug metabolizing enzymes, collectively called the Ah locus. The regulation of induction of these enzymes has been studied most extensively in the mouse. The genetic responsiveness to PAH induction of AHH activity has been correlated strongly with the presence of a cytosolic binding protein. This binding protein has been shown to bind TCDD and PAHs reversibly and with high affinity. Analysis of the binding proteins indicates that there are at least two distinct high-affinity binding proteins which might play a role in the regulation of expression of AHH activity. Several investigators have reported a 4 - 5.5 S protein that displays saturable high affinity binding to substrates such as 3-methylcholanthrene and benzo(a)pyrene, whereas others have described an 8 - 9 S protein that displays saturable, high-affinity binding to TCDD and a variety of PAHs. The potential role of the "4S" binding protein in the regulation of cytochrome P-450c expression in the rat was investigated in one study. Using ion-exchange, gel permeation and hydrophobic

interaction chromatography, the protein was purified over 6,000-fold from rat liver cytosol. This binding protein was shown to differ from the 9S binding protein characterized in other laboratories. Under high stringency conditions this highly purified 3.3 S binding protein was shown to interact in a specific and saturable manner with several subclones of the rat cytochrome P-450 gene containing 5' upstream sequences, as well as portions of intron 1. Binding was not observed to the coding portions of the gene. These data implicate the "4S" binding protein in the transregulation of rat cytochrome P-450c expression (22).

Role of DNA Repair in Carcinogenesis: The types of projects in this subject area include studies on the characterization of DNA damage produced by bulky chemical carcinogens, alkylating agents, UV light and ionizing radiation; the isolation and characterization of proteins responsible for DNA nucleotide excision repair and base excision repair; the cloning and characterization of the DNA nucleotide and base excision repair genes; and the determination of the role of chromosome structure, location and site of DNA lesions and poly (ADP-ribosylation) in the repair of DNA damage. A variety of rodent, frog, yeast, bacterial and normal and repair-deficient human cells are being used in these studies. Since DNA modification by chemical carcinogens has been shown to lead to mutations or other alterations in gene expression, DNA repair may have evolved as a defense mechanism to eliminate such damage and thus restore the correct genetic information and DNA structure. The significance of this process to human well being was emphasized by the demonstration that, in contrast to normal cells, cells from individuals with xeroderma pigmentosum, a hereditary genetic disease, did not remove UV radiation-induced pyrimidine dimers from their DNA. These individuals develop multiple skin cancers at an early age only on exposed surfaces, thus establishing a link between genetic damage, defective repair of the damage, and cancer in the exposed tissue.

O⁶-Methylguanine is one of the products produced by carcinogenic and mutagenic methylating agents in DNA. It is known that this lesion is repaired by the action of a protein, O⁶-alkylguanine-DNA alkyltransferase (AGT), which catalyzes the stoichiometric transfer of the methyl group to a cysteine residue within its own sequence restoring the guanine in DNA. It has been shown that this protein is not specific for methyl groups since the AGTs from many animal and human tissues and *E. coli* act on O⁶-ethylguanine and on hydroxyethyl groups. In order to determine whether longer chain alkyl groups serve as substrates for the AGT, DNA substrates containing O⁶-n-butylguanine, O⁶-iso-butylguanine, O⁶-n-propylguanine and O⁶-iso-propylguanine were prepared. These substrates were used to test the ability of AGTs from *E. coli* and rat liver to remove such alkyl groups from the O⁶-position of guanine. All of the adducts were shown to be removed by the AGTs, but the branched alkyl chain iso-butyl and iso-propyl adducts were removed very slowly. As the size of the alkyl group increased, the reaction rate was shown to decrease. There were, however, significant differences in relative rates between the rat liver and *E. coli* AGTs. The rat liver AGT repaired O⁶-methylguanine more slowly than the *E. coli* protein, but was considerably more rapid than the bacterial equivalent when acting on n-propyl and n-butyl adducts. The results obtained indicated that differential rates of repair may contribute to the relative risks of carcinogenesis and mutagenesis by exposure to alkylating agents of different size and that rates of repair may be species-specific and must be determined from specific measurements rather than extrapolated from data on other organisms. This is further illustrated by the demonstrated differences in the ability of the rat liver and *E. coli* AGTs to remove methyl groups from another O-alkylated DNA base, O⁴-methylthymine. A calf thymus DNA substrate was used in which O⁴-methylthymidine represented 0.06% of the total methylation. When the methylated DNA substrate was incubated with an excess of either of the AGTs,

greater than 95% of the O⁶-methyldeoxyguanine was removed. The *E. coli* AGT was shown to remove 89% of the O⁴-methylthymidine, but the rat liver AGT did not alter the content of O⁴-methylthymidine. These results indicate that the mammalian AGT is specific for O⁶-methylguanine and differs from the bacterial protein in that it does not demethylate O⁴-methylthymine at any significant rate (171).

The frequency of transformation of mouse cells by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and other mutagenic compounds has been shown to be greater in synchronously proliferating cells than in confluent cultures. The dependence of malignant transformation on cell proliferation of carcinogen-treated cells both in vivo and in vitro has prompted the study of DNA damage and repair as a function of cell cycle. Preferential methylation of replicating sites and lack of repair of O⁶-methylguanine lesions in DNA has been reported in C3H 10T1/2 cells during S-phase. It is possible that the lack of repair of O⁶-methylguanine in S-phase cells may be due to either a loss of O⁶-methylguanine DNA methyltransferase (AGT) or to changes in the chromatin structure which would make the DNA inaccessible to the repair enzyme. In order to test one of the possibilities, the level of AGT was measured in partially synchronized cultures of C3H 10T1/2 cells as a function of cell cycle. The AGT level was significantly reduced prior to the onset of S-phase. This reduction was concomitant with the inhibition of in vivo repair of O⁶-methylguanine in DNA of S-phase cells as observed earlier. The recovery of the AGT level was observed to parallel the progression of synchronized cells into G₂. The amount of AGT present in S-phase cells was shown to be about 15% of the levels present in G₀ or early G₁ cells. A comparison of the in vivo repair of O⁶-methylguanine and AGT levels suggests that the lack of repair of O⁶-methylguanine in DNA of C3H 10T1/2 cells is due only in part to a temporal loss of AGT (159).

The UV component of sunlight is thought to have a substantial impact on human health because it is thought to be the agent responsible for the induction of most skin cancers. A variety of experiments have been performed investigating the biological significance of dimers in cells exposed to solar UV wavelengths (greater than 290 nm wavelengths). The results have demonstrated that damage other than cyclobutane pyrimidine dimers plays a critical role in solar UV-irradiated cells. It is therefore of importance to investigate the mechanisms by which these solar UV-induced nondimer DNA lesions are repaired. The bromodeoxyuridine photolysis assay was used to examine the excision repair of solar UV-induced nondimer damage in ICR 2A frog cells in one study. Cultures of cells were also exposed to 254 nm UV light, which induces primarily dimers, and ⁶⁰Co gamma rays. It was found that the solar UV-induced nondimer damage was repaired by a short patch repair mechanism in which less than about 20 nucleotides are inserted into a repaired region. Similar results were also obtained for gamma-irradiated cells. In contrast, excision repair of 254 nm-induced dimers was accomplished by a long patch process in which an average of about 180 nucleotides are inserted into the repaired sites. In a further study, the ability of ICR 2A frog and normal human skin fibroblasts to repair pyrimidine dimers and nondimer DNA damage was compared. The results showed that the excision repair capacity of human cells was about 200-fold higher than that of frog cells. Also, the rate of excision repair in solar UV-irradiated cells was shown to be about one third the level detected in 254 nm-irradiated cells. This result is consistent with the conclusion that the pathway(s) involved in the repair of solar UV-induced DNA damage differs from the repair of lesions produced in cells exposed to 254 nm UV light (187).

MOLECULAR CARCINOGENESIS
GRANTS ACTIVE DURING FY86

<u>Investigator/Institution/Grant Number</u>	<u>Title</u>
1. ACS, George Mount Sinai School of Medicine 5 R01 CA 16890-09	Studies on Chemotherapeutic Deoxyribonucleosides
2. ADAIR, Gerald M. Univ. of Texas System Cancer Center 5 R01 CA28711-05	Expression of Genetic Variation in Cultured Cells
3. ALBERTINI, Richard J. Univ. of Vermont & St. Agric. College 2 R01 CA30688-04A1	Direct Mutagenicity Testing in Man
4. AMES, Bruce N. University of California (Berkeley) 1 R35 CA39910-01	Mutagenesis and Carcinogenesis
5. ASHENDEL, Curtis L. Purdue University West Lafayette 5 R01 CA36262-03	Interactions of Tumor Promoters with Receptors
6. AVADHANI, Navayan G. University of Pennsylvania 2 R01 CA22762-09	Mitochondrial DNA Damage during Chemical Carcinogenesis
7. BAIRD, William M. Purdue University West Lafayette 1 R01 CA40228-01	Molecular Mechanisms of Hydro- carbon DNA Interactions
8. BAXTER, C. Stuart University of Cincinnati 5 R01 CA34446-02	In Vivo Immunotoxicology of Tumor- Promoting Agents
9. BAXTER, C. Stuart University of Cincinnati 5 R01 CA36183-02	Tumor Promotion by Environmental N Alkanes
10. BECKER, Frederick F. Univ. of Texas System Cancer Center 5 R01 CA20657-10	Phenotypic Analysis of Chemical Carcinogenesis
11. BECKER, Frederick F. Univ. of Texas System Cancer Center 5 R01 CA28263-06	Chromosomal Proteins during Chemical Carcinogenesis
12. BENFIELD, John R. City of Hope National Medical Center 5 R01 CA29373-05	Model of Bronchogenic Lung Cancer

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| 13. BERRY, David L.
U.S. Agriculture Research Service
5 R01 CA 28968-03 | Mode of Action of Phorbol Esters
in Epidermal Cells |
| 14. BIRT, Diane F.
University of Nebraska Medical Center
5 R01 CA33368-03 | Urinary Bladder Cancer Promotion
by Dietary L-Tryptophan |
| 15. BOUCK, Noel P.
Northwestern University
2 R01 CA27306-07 | Genetic Analysis of Malignant
Transformation |
| 16. BOWDEN, George T.
University of Arizona
1 R01 CA40584-01 | Oncogene Activation during Skin
Tumor Progression |
| 17. BOX, Harold C.
Roswell Park Memorial Institute
5 R01 CA29425-06 | Molecular Studies of Carcino-
genesis and Mutagenesis |
| 18. BOYNTON, Alton L.
University of Hawaii at Manoa
1 R01 CA39745-01 | Assays for and Mechanisms of
Action of Tumor Promoters |
| 19. BRASITUS, Thomas A.
Michael Reese Hospital and
Medical Center (Chicago)
5 R37 CA36745-04 | Colonic Epithelial Cell Plasma
Membranes |
| 20. BRESNICK, Edward
University of Nebraska Medical Center
2 R01 CA36679-04 | DNA Repair after Polycyclic
Hydrocarbon Administration |
| 21. BRESNICK, Edward
University of Nebraska Medical Center
5 R01 CA35994-03 | Epoxide Hydrolase in Hyperplastic
and Neoplastic Livers |
| 22. BRESNICK, Edward
University of Nebraska Medical Center
5 R01 CA36106-04 | Polycyclic Hydrocarbon Metabolism
and Carcinogenesis |
| 23. BROUDE, Suse B.
New York University
5 R01 CA28038-06 | Carcinogen - DNA Adducts: Linkage
Site and Conformation |
| 24. BUTEL, Janet S.
Baylor College of Medicine
5R01 CA 33369-03 | Tumor Promotion and Murine Mammary
Cancer |
| 25. BYUS, Craig V.
University of California (Riverside)
5 R01 CA35807-02 | Mechanism of Tumor-Promoter Action |

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| 26. CALOS, Michele P.
Stanford University
2 R01 CA33056-04 | Mutation in Human Cells at the DNA
Sequence Level |
| 27. CARTER, Timothy H.
St. John's University
5 R01 CA37761-02 | Regulation of Transcription by a
Tumor Promoter |
| 28. CHANG, Ching-Jer
Purdue University West Lafayette
5 R01 CA35904-03 | Chemical Carcinogens and DNA Inter-
actions in Tissue Culture |
| 29. CHRISTMAN, Judith K.
Mount Sinai School of Medicine
5 R01 CA25985-07 | Response of Phagocytic Leukocytes
to Tumor Promoters |
| 30. CLARKSON, Judith M.
Univ. of Texas System Cancer Center
5 R01 CA 19281-09 | Cell-Cycle Related DNA Repair
Mechanisms |
| 31. CLAWSON, Gary A.
Univ. of California (San Francisco)
1 R01 CA40145-01 | Specificity of RNA Transport
In Vitro |
| 32. CONNER, Mary K.
University of Pittsburgh
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| 33. CONTI, Claudio J.
University of Texas
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| 34. CORDEIRO-STONE, Marila
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| 35. COSTA, Max
New York University
5 R01 CA43070-02 | Mechanism of Metal Carcinogenesis |
| 36. CRAIGHEAD, John E.
Univ. of Vermont & St. Agric. College
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| 37. CURPHEY, Thomas J.
Dartmouth College
2 R01 CA30650-04A1 | Pancreas and Liver Carcinogen
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| 38. CZECH, Michael P.
Univ. of Massachusetts Medical School
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| 39. DANIELSON, Keith G.
Baylor College of Medicine
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40. DAVIDSON, Richard L.
University of Illinois at Chicago
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41. DETRISAC, Carol J.
IIT Research Institute
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42. DIAMOND, Leila
Wistar Institute of Anatomy and Biology
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43. DIAMOND, Leila
Wistar Institute of Anatomy and Biology
5 R01 CA23413-08
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44. DIGIOVANNI, John
Univ. of Texas System Cancer Center
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45. DIGIOVANNI, John
Univ. of Texas System Cancer Center
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46. DRESLER, Steven L.
Washington University
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47. DRINKWATER, Norman R.
University of Wisconsin (Madison)
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48. DUFFEL, Michael W.
University of Iowa
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49. DUKER, Nahum J.
Temple University
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50. ECHOLS, G Harrison, Jr.
University of California (Berkeley)
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51. ESTENSEN, Richard D.
Univ. of Minnesota of (Mnpls-St Paul)
5 R01 CA22195-08
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52. ETHIER, Stephen P.
Michigan Cancer Foundation
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53. FAHL, William E.
University of Wisconsin (Madison)
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54. FAHL, William E.
University of Wisconsin (Madison)
5 R01 CA42066-02 Hydrocarbon Carcinogenesis in
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56. FARBER, John L.
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5 R01 CA 32610-04 Hepatocarcinogenesis: A Role for
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Brown University
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58. FELDBERG, Ross S.
Tufts University
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59. FINE, David H.
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60. FINK, Gerald R.
Whitehead Institute for Biomedical Res.
5 R01 CA39961-03 Chemical Carcinogens and Frame-
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61. FISCHER, Susan M.
Univ. of Texas System Cancer Center
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62. FISCHER, Susan M.
Univ. of Texas System Cancer Center
1 R01 CA42211-01 Tumor Promoter-Induced Oxidants
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63. FISHER, Paul B.
Columbia University (New York)
5 R01 CA35675-03 Analysis of Progression of the
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64. FOILES, Peter
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1 R01 CA42235-01 Monoclonal Antibodies Specific for
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65. FOSTER, Patricia L.
Boston University
5 R01 CA37880-02 Mechanisms of Mutagenesis by
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66. FOX, C. Fred
University of California
1 R13 CA42086-01 Conference on DNA Replication and
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67. FRENKEL, Krystyna
New York University
5 R01 CA37858-02 Tumor Promoters Effecting Base
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| 68. FRIEDBERG, Errol C.
Stanford University
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| 69. GARTE, Seymour J.
New York University
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| 70. GEACINTOV, Nicholas E.
New York University
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| 71. GOLD, Barry I.
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| 72. GOULD, Michael N.
University of Wisconsin (Madison)
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| 73. GOULD, Michael N.
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| 74. GREENBERGER, Joel S.
Univ. of Massachusetts Medical School
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| 75. GRIFFITH, O. Hayes
University of Oregon
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Univ. of North Carolina Chapel Hill
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| 77. GRISHAM, Joe W.
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| 78. GRISHAM, Joe W.
Univ. of North Carolina Chapel Hill
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| 79. GROLLMAN, Arthur P.
State University New York (Stony Brook)
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| 80. GRUNBERGER, Dezider
Columbia University (New York)
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| 81. GUDAS, Lorraine J.
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Vanderbilt University
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83. GUPTA, Pawan K.
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84. GUPTA, Ramesh C.
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85. GURTOO, Hira L.
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86. HAM, Richard G.
University of Colorado at Boulder
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87. HANKINSON, Oliver
University of California (Los Angeles)
2 R01 CA28868-07
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88. HASELTINE, William A.
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89. HASELTINE, William A.
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90. HECHT, Stephen S.
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91. HEIN, David W.
Morehouse School of Medicine
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92. HENDERSON, Earl E.
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93. HENDRICKS, Jerry D.
Oregon State University
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94. HITTELMAN, Walter N.
Univ. of Texas System Cancer Center
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95. HITTELMAN, Walter N.
Univ. of Texas System Cancer Center
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Rhode Island Hospital (Providence, RI)
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97. HIXSON, Douglas C.
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98. HNILICA, Lubomir S.
Vanderbilt University
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Vanderbilt University
5 R01 CA36479-02 Nuclear Toxicity of Heavy Metals
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Princeton University
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101. HOLMES, Eric H.
Pacific Northwest Research Foundation
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102. HOSEIN, Barbara H.
New York Blood Center
5 R23 CA 34621-03 Human Epidermal Differentiation
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103. HOWARD-FLANDERS, Paul
Yale University
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104. HUMAYUN, M. Zafri
Univ. of Medicine & Dentistry of NJ
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105. HUNT, John M.
Univ. of Texas Hlth. Sci. Ctr. (Houston)
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106. HUTCHINSON, Franklin
Yale University
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107. IP, Margot M.
Roswell Park Memorial Institute
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108. ISSENBERG, Phillip
University of Nebraska Medical Center
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109. IVARIE, Robert D.
University of Georgia
5 R01 CA34066-03 Inactivation of Gene Expression
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| 110. JACOBSON, Myron K.
Texas College of Osteopathic Medicine
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| 111. JENSEN, David E.
Temple University
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Duke University
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University of Southern California
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| 114. JUNGALWALA, Firoze B.
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University of Pennsylvania
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Univ. of North Carolina Chapel Hill
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Univ. of North Carolina Chapel Hill
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| 118. KAUFMAN, David G.
Univ. of North Carolina Chapel Hill
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| 119. KAUFMANN, William K.
Univ. of North Carolina Chapel Hill
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| 120. KENNEDY, Ann R.
Harvard University
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| 121. KLEIN-SZANTO, Andres J.
Univ. of Texas System Cancer Center
5 R01 CA38863-02 | Markers of Skin Tumor Progression |
| 122. KLEIN-SZANTO, Andres J.
Univ. of Texas System Cancer Center
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| 124. KOESTNER, Adalbert
Michigan State University
5 R01 CA32594-04 | Neurooncogenesis by Resorptive Carcinogens |
| 125. KOHWI-SHIGEMATSU, Terumi
La Jolla Cancer Research Foundation
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| 126. KOLESNICK, Richard N.
Sloan-Kettering Institute Cancer Res.
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University of Alabama at Birmingham
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Yeshiva University
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| 129. KRUGH, Thomas R.
University of Rochester
5 R01 CA35251-03 | Mechanism of Action of Carcinogens |
| 130. KULESZ-MARTIN, Molly
Roswell Park Memorial Institute
5 R01 CA31101-05 | Quantitative Carcinogenetics in Epithelial Cell Lines |
| 131. LALWANI, Narendra D.
Northwestern University
5 R23 CA38196-02 | Receptors for Carcinogenic Peroxisome Proliferators |
| 132. LAPEYRE, Jean-Numa
Univ. of Texas System Cancer Center
5 R01 CA31487-05 | Regulation and Enzymology of DNA Methylase in Cancer |
| 133. LEADON, STEVEN A.
University of California
Lawrence Berkeley Laboratory
1 R01 CA40453-01 | DNA Repair in Specific Sequences of Mammalian Cells |
| 134. LEGERSKI, Randy J.
Univ. of Texas System Cancer Center
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| 135. LIEBERMAN, Michael W.
Fox Chase Cancer Center
5 R01 CA39392-03 | Carcinogen Activation of Unexpressed Mammalian Genes |
| 136. LIEBERMAN, Michael W.
Fox Chase Cancer Center
5 R01 CA40263-02 | Gene Expression in Carcinogen-Induced Liver Cancer |

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Yeshiva University
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138. LINDAHL, Ronald G.
University of Alabama in University
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139. LOEB, Lawrence A.
University of Washington
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140. LOMBARDI, Benito
University of Pittsburgh
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141. LOMBARDI, Benito
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142. MACLEOD, Michael C.
Univ. of Texas System Cancer Center
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143. MACMANUS, John P.
National Research Council of Canada
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144. MAGUN, Bruce E.
University of Arizona
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145. MAHER, Veronica M.
Michigan State University
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146. MAHER, Veronica M.
Michigan State University
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147. MAHER, Veronica M.
Michigan State University
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148. MALKINSON, Alvin M.
University of Colorado at Boulder
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149. MARCHOK, Ann C.
Oak Ridge National Laboratory
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150. MARLETTA, Michael A.
Massachusetts Institute of Technology
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151. MARNETT, Lawrence J.
Wayne State University
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152. MAY, William S.
Johns Hopkins University
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153. MC CORMICK, J. Justin
Michigan State University
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154. MEEHAN, Thomas
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155. MEHLMAN, Myron A.
Mount Sinai School of Medicine
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156. MICHALOPOULOS, George K.
Duke University
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157. MILLER, Elizabeth C.
University of Wisconsin (Madison)
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158. MILO, George E.
Ohio State University
5 R01 CA25907-06
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159. MITRA, Sankar
Oak Ridge National Laboratory
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160. MOORE, Peter D.
University of Illinois at Chicago
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161. MORDAN, Lawrence J.
University of Hawaii at Manoa
1 R01 CA38806-01A1
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162. MORIZOT, Donald C.
Univ. of Texas System Cancer Center
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163. MOSES, Robb E.
Baylor College of Medicine
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164. MOSSMAN, Brooke T.
Univ. of Vermont & St. Agric. College
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| 165. NGUYEN-HUU, Chi
Columbia University (New York)
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| 166. O'BRIEN, Thomas G.
Wistar Institute of Anatomy and Biology
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| 167. OBERLEY, Larry W.
University of Iowa
1 R01 CA41267-01 | Superoxide Dismutase Levels in Tumor
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| 168. OYASU, Ryoichi
Northwestern University
2 R01 CA33511-04A1 | Experimental Urinary Bladder
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| 169. PALL, Martin L.
Washington State University
5 R01 CA 33503-03 | Tandem Gene Duplication and
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| 170. PARSA, Ismail
Health Science Center at Brooklyn
1 R01 CA38955-01A1 | Quantitative Model of Human Pancreas
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| 171. PEGG, Anthony E.
Pennsylvania State University-
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5 R01 CA18137-11 | Persistence of Alkylated DNA in
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| 172. PELLING, Jill C.
University of Nebraska Medical Center
5 R01 CA40847-02 | Two-Stage Skin Carcinogenesis and
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| 173. PENMAN, Sheldon
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5 R01 CA37330-02 | Cytoarchitecture in Tumor Promotion
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| 174. PERCHELLET, Jean-Pierre H.
Kansas State University
1 R01 CA40083-01 | Glutathione Metabolism during Skin
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| 175. PIZER, Lewis I.
University of Colorado
1 R01 CA42444-01 | Mutagenic Activity of Anti-Herpes
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| 176. POUR, Parviz M.
University of Nebraska Medical Center
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| 177. RANDERATH, Kurt
Baylor College of Medicine
5 R01 CA32157-05 | 32P-Labeling Test for Nucleic Acid
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| 178. RAO, M Sambasiva
Northwestern University
5 R01 CA36130-02 | Gamma-Glutamyltranspeptidase
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194. SCARPELLI, Dante G.
Northwestern University
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195. SEDWICK, W. David
Duke University
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196. SHARMA, Surendra
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197. SHAY, Jerry W.
Univ. of Texas Hlth. Sci. Ctr. (Dallas)
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198. SHIM, Sang C.
Korea Advanced Institute of Science and Technology
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199. SHINOZUKA, Hisashi
University of Pittsburgh
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200. SHINOZUKA, Hishashi
University of Pittsburgh
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201. SIDRANSKY, Herschel
George Washington University
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202. SINGER, Bea A.
University of California-Lawrence Berkeley Laboratory
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203. SIRICA, Alphonse E.
Virginia Commonwealth University
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204. SIROVER, Michael A.
Temple University
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205. SMUCKLER, Edward A.
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| 180. REDDY, Arram L.
University of Washington
5 R01 CA32716-03 | Skin Tumorigenesis Studied with
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| 181. RICH, Alexander
Massachusetts Institute of Technology
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| 182. RICHIE, Ellen R.
Univ. of Texas System Cancer Center
5 R01 CA37912-02 | Mechanisms of MNU-Induced Lymphoma |
| 183. ROGAN, Eleanor G.
University of Nebraska Medical Center
5 R01 CA25176-05 | Binding of Aromatic Hydrocarbons
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| 184. ROMANO, Louis J.
Wayne State University
1 R01 CA40605-01A1 | Biological Consequences of Site-
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| 185. ROMANO, Louis J.
Wayne State University
5 R01 CA35451-03 | In Vitro Function of DNA Containing
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| 186. RONEN, Amiram
Hebrew University of Jerusalem
1 R01 CA40922-01 | A Direct Test for Somatic Mutation
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| 187. ROSENSTEIN, Barry S.
Univ. of Texas Hlth. Sci. Ctr. (Dallas)
2 R01 CA33920-04 | Repair of 290-320 Induced Non-Dimer
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| 188. ROSNER, Marsha R.
Massachusetts Institute of Technology
5 R01 CA35541-03 | Modulation of Cellular Phosphory-
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| 189. ROSNER, Marsha R.
Massachusetts Institute of Technology
1 R01 CA40407-01 | Regulation of Protein Kinase C |
| 190. ROSSMAN, Toby G.
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| 191. ROSSMAN, Toby G.
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5 R01 CA35631-03 | Carcinogen-Mediated Genetic Effects |
| 192. SARMA, D. S.
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207. SOLT, Dennis B.
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208. SOROF, Sam
Institute for Cancer Research
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209. STAMBROOK, Peter J
University of Cincinnati
5 R01 CA36897-02
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210. STONER, Gary D.
Medical College of Ohio at Toledo
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211. STRAUSS, Bernard S.
University of Chicago
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University of Chicago
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213. SUDILOVSKY, Oscar
Case Western Reserve University
5 R01 CA35362-02
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214. SUMMERS, William C.
Yale University
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215. TEEBOR, George W.
New York University
5 R01 CA16669-11
Repair of Radiation-Induced Carcino-
genic Damage to DNA
216. TERZAGHI-HOWE, Margaret
Oak Ridge National Laboratory
5 R01 CA34695-03
Cell Interactions: Expression of
Preneoplastic Markers
217. TOPAL, Michael D.
Univ. of North Carolina Chapel Hill
5 R01 CA28632-06
Effects of Carcinogen Modification
of DNA Precursors
218. TROSKO, James E.
Michigan State University
5 R01 CA21104-09
Mutation and Derepression of Genes
in Carcinogenesis
219. TUKEY, Robert H.
University of California (San Diego)
5 R01 CA37139-03
Cytochrome P-450 Genes and Chemical
Carcinogenesis

220. VERMA, Ajit K.
University of Wisconsin (Madison)
5 R01 CA35368-03
CA2+-Dependent Processes Involved
in Phorbol Ester Tumor Promotion
221. WALDSTEIN, Evelyn A.
Tel Aviv University
5 R01 CA35895-03
Regulation of Induced O6-Methyl-
guanine Repair in Cells
222. WALKER, Graham C.
Massachusetts Institute of Technology
5 R01 CA21615-10
Mutagenesis and Repair of DNA
223. WEBBER, Mukta M.
Univ. of Colorado Hlth. Sci. Ctr.
5 R01 CA33169-03
Intrinsic and Extrinsic Tumor
Promoters in Prostate Cancer
224. WEBER, Wendell W.
University of Michigan at Ann Arbor
5 R01 CA39018-02
N-Acetylation Pharmacogenetics:
Arylamines and DNA Damage
225. WEINSTEIN, I. Bernard
Columbia University (New York)
5 P01 CA21111-09
Molecular Events in Chemical
Carcinogenesis
226. WEINSTEIN, I. Bernard
Columbia University (New York)
5 R01 CA26056-07
Cellular and Biochemical Effects
of Tumor Promoters
227. WEISSMAN, Bernard E.
Children's Hospital of Los Angeles
1 R01 CA39602-01A1
Chemical Transformation of Mouse
Epidermal Keratinocytes
228. WENDER, Paul A.
Stanford University
5 R01 CA31841-05
Synthetic Studies on Tumor Promoters
and Inhibitors
229. WENNER, Charles E.
Roswell Park Memorial Institute
5 R01 CA13784-12
The Effect of Cocarcinogens on
Cellular Membranes
230. WETTERHAHN, Karen E.
Dartmouth College
5 R01 CA34869-03
Interaction of Chromate with
Mitochondria
231. WHALEN, Dale L.
Univ. of Maryland Balt. Co. Campus
5 R01 CA17278-08
Kinetic Studies of Aryl Epoxide
Reactions
232. WHITLOCK, James P., Jr.
Stanford University
2 R37 CA32786-04
Carcinogen-Metabolizing Enzymes:
Action in Variant Cells
233. WILLIAMS, Gary M.
American Health Foundation
1 R01 CA39545-01A1
Biochemical Toxicity of Agents
Increasing Reactive O2

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| 234. WILLIAMS, Jerry R.
Johns Hopkins University
7 R01 CA 39531-01 | Cellular Hypermutability in Cancer Promotion |
| 235. WINKLE, Stephen A.
Rutgers The State Univ. (New Brunswick)
5 R01 CA34762-03 | Cooperative, Selective Carcinogen, Drug Binding to DNA |
| 236. WITSCHI, Hanspeter R.
Oak Ridge National Laboratory
5 R01 CA 33795-03 | Enhancement of Lung Tumor Formation: Cell Kinetics |
| 237. WITZ, Gisela
Univ. of Medicine & Dentistry NJ
5 R01 CA33270-03 | Free Radicals in Tumor Promotion |
| 238. YAGER, James D., Jr.
Dartmouth College
2 R01 CA36713-04 | DNA Sequence Changes during Hepato-carcinogenesis |
| 239. YAGER, James D., Jr.
Dartmouth College
5 R01 CA36701-03 | Role of Gonadal Steroids in Hepato-carcinogenesis |
| 240. YAMASAKI, Hiroshi
World Health Organization - International Agency Research on Cancer
1 R01 CA40534-01 | Role of Intercellular Communication in Carcinogenesis |
| 241. YANG, Nien-Chu C.
University of Chicago
5 R01 CA 10220-15 | Chemistry of Biologically Active Oxiranes |
| 242. YU, Fu-Li
College of Medicine at Rockford
5 R01 CA30093-05 | Aflatoxin B ₁ and Nucleolar RNA Synthesis |
| 243. ZURLO, Joanne
Dartmouth College
5 R23 CA36782-03 | Inducible DNA Repair in Pancreatic Carcinogenesis |

SUMMARY REPORT

SMOKING & HEALTH

The Smoking and Health component within the Chemical and Physical Carcinogenesis Branch includes 10 grants with FY86 funding of \$1,879,531 million and two contracts with FY86 funding of \$0.42 million. It continues to support research directed toward understanding and mitigating the deleterious effects of smoking on health. Significant past efforts have included identifying related smoking diseases and chemical analyses of major whole smoke components and their subsequent metabolic products. Current program activities are focused on the toxicological and pharmacological aspects of the problem, with emphasis on nicotine and nicotine metabolites, and their effect on smoke dosimetry in humans.

Smoking constitutes the principal preventable risk factor for cancer, as well as for a host of other diseases. In view of this fact, surprisingly little is known about what makes this habit so compelling that nearly a third of the U.S. population continues to smoke despite an extensive public education campaign spanning more than two decades. Lung cancer has been directly associated with cigarette smoking and successful treatment at this site is less than that for many forms of cancer. One ongoing study (9) supports research methodology that has involved the simulation of key factors in the stimulus control of smoking behavior in a laboratory setting. Examples include the use of exteroceptive cues such as cigarette availability and interoceptive cues such as psychological stress as well as manipulations of nicotine dosage/deprivation and different routes of administration. Dependent measures have included smoking topography, plasma cotinine and nicotine, task performance, subjective states such as anxiety, pain, and alertness, cardiac rate and peripheral body temperature, and neuroendocrine responses, including catecholamines and neuropeptides. Blockers of nicotine cholinergic and endogenous opioid activity (mecamylamine and naloxone) have been used in order to establish causal links between pharmacological actions of nicotine and concomitant subjective and physiological effects. Results reported to date include: 1) a clear-cut demonstration of nicotine dose-related antinociception in minimally-deprived smokers and in ex-smokers, casting doubt on the theory that reinforcement for smoking is based principally upon relief of nicotine withdrawal; 2) establishment of nicotine thresholds for stimulation of nine pituitary hormones, replicating and extending the previous observation of beta-endorphin release in response to nicotine from smoking; 3) a demonstration of increased smoking under conditions of anxiety induced by a psychological stressor (mental arithmetic), indicating a relationship between stress and nicotine intake and suggesting a possible role for nicotine-stimulated beta-endorphin release; 4) a comprehensive, testable biobehavioral explanation of reinforcement in smoking that incorporates both relief of nicotine withdrawal and nicotine stimulation of neuroregulators with behavioral and subjective effects. Continuing work in this study is currently exploring the contribution of endogenous opioid activity to the antinociceptive and anxiolytic effects of nicotine in both smokers and nonsmokers.

A second study on smoking behavior in the human is continuing (11). Smoking behaviors such as puff and inhalation volumes are thought to play a critical role in determining tobacco smoke exposure, but no previous studies have systematically manipulated smoking behaviors to experimentally determine their effects on smoke uptake in humans. In a series of three experiments, one of the following smoking characteristics was manipulated, while the other two were held constant: 1) puff volume (15, 30, 45, and 60 ml), 2) inhalation volume (0, 20, 40, and 60% of vital

capacity) and 3) breathhold duration (0, 4, 8, and 16 sec). A critical prerequisite to this experimental approach was the development of a computer-controlled auditory feedback system that provided real-time measurement and control of each smoking behavior component. When the smoke dose was manipulated by changing puff volume, blood nicotine and expired breath carbon monoxide levels also changed in an orderly fashion. Breathhold duration influenced carbon monoxide, but not nicotine blood levels. It appeared that all the available nicotine from each smoke puff was extracted within the first few seconds of lung exposure. Finally, inhalation volume (the amount of air mixed with the smoke dose) had no effect on either nicotine or carbon monoxide levels. The conclusion drawn from these studies was that puff volume is an important determinant of smoke exposure, but that inhalation components of smoking behavior have little influence on exposure levels. Additional experiments conducted under this study have determined the conditions under which smokers adjust their puffing behavior to compensate for changes in smoking availability and cigarette characteristics. In one experiment, smoke dilution was manipulated by placing cellophane tape around all or part of the filter ventilation holes of ultra low-yield cigarettes. Subjects took more puffs and longer puffs from regular filter vented cigarettes than from the same cigarettes with filter vents blocked. These behavioral changes suggest that smokers were attempting to compensate for smoke dilution caused by filter venting. In a second experiment, subjects smoked University of Kentucky Reference Cigarettes that differed in nicotine delivery but were identical in tar delivery and in taste and draw characteristics. Puff volumes were larger when subjects smoked low as compared with high nicotine yield cigarettes. In a final experiment, smoking deprivation was manipulated and effects on smoking behavior observed. Subjects were allowed to smoke their own cigarettes as they wished for one hour following 3, 30 or 300 min. of smoking deprivation. Subjects smoked an extra cigarette and took more puffs during the hour following a long (300 min) deprivation period, but puff volumes were not changed. These studies suggest that puff volume is likely to change only when cigarette characteristics, such as smoke dilution or nicotine yield, are altered. Overall, these studies are contributing to the knowledge of how detailed cigarette smoking behaviors contribute to smoke exposure and thus to the health risks of cigarette smoking.

The increased use of clove cigarettes (Kreteks) in the United States is reflected in a 40% higher export rate of this modified Indonesian tobacco product to the United States in 1985 when compared with 1984. Coupled with this increase in consumption, there have been several reports of adverse health effects associated specifically with the smoking of clove cigarettes, including the occurrence of pulmonary edema, bronchospasms, and hemoptysis. These facts prompted studies (5) to assess the relative differences in both composition and toxicity of the smoke components of Kreteks (approximately 40% cloves and 60% tobacco). A major constituent in the smoke of clove cigarettes is eugenol (9-16 mg/cig). In addition, the smoke of clove cigarettes contains significant amounts of eugenol acetate, alpha-humulene, beta-caryophyllene, and possibly, beta-caryophyllene oxide. It was suspected that acute toxicity of these compounds might be a reason for some of the adverse health effects observed after smoking clove cigarettes. Yet, inhalation of smoke from Kreteks in exposure devices used for assaying cigarette smoke did not lead to acute toxicity in Syrian golden hamsters. This is attributed, in part, to the fact that exposure of the animals is limited to the upper respiratory airways due to their shallow breathing in the apparatus. In addition to this observation, the potential toxic effects of the suspect compounds have been evaluated by intratracheal instillation. Eugenol proved to be highly toxic when administered intratracheally, having an LD₅₀ of 11 mg/kg in male F344 rats and 17 mg/kg in male Syrian golden hamsters. Congestion of the lung and

interstitial hemorrhages, acute emphysema, and acute pulmonary edema were among the macroscopic and histologic findings observed. In view of the smoke deliveries of eugenol reported for several brands of Kreteks, these observations raise great concerns with regard to possible human health effects. Further studies are in progress to develop more efficient inhalation exposure in animals to clove cigarette smoke and its major components and to fully assess the acute and chronic effects of such exposure.

The Smoking and Health Program continues to support work on the health effects of tobacco smoke components and more recently on the smokeless tobacco chemical constituents. Emphasis is being placed on a group of compounds (4) referred to as tobacco-specific N-nitrosamines (TSNA). The major objective of one study is to understand how the carcinogenic tobacco-specific nitrosamines N'-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) interact with DNA and initiate the carcinogenic process. In the past year, research has focused on NNK because of its high carcinogenic activity. A comparative investigation of tumorigenicity and DNA methylation by NNK and N-nitrosodimethylamine (NDMA), a well-known carcinogenic nitrosamine, was carried out in F344 rats. In the tumorigenicity study, each rat received a total dose of 0.3 mmol/kg of NNK or NDMA, administered by s.c. injection over a period of 20 weeks. The experiment was terminated after 104 weeks. The results showed that NNK was a more potent tumorigen than NDMA under these conditions since NNK induced significantly more lung tumors and nasal cavity tumors than did NDMA. Both nitrosamines gave similar yields of liver tumors. For the comparative DNA methylation study, groups of rats were treated by s.c. injection with either NNK or NDMA and levels of 7-methylguanine and 8-methylguanine was measured in liver, lung, and nasal mucosa 1-48 h after treatment. In liver and lung, levels of 7-methylguanine and 8-methylguanine were significantly greater in NDMA than in NNK-treated rats; levels of methylation in the nasal mucosa were similar. Since NNK was more tumorigenic than NDMA, the results suggest that factors other than, or in addition to, DNA methylation are important in NNK tumorigenesis. One of the other factors is believed to be formation of pyridyloxobutyl adducts in DNA by NNK. This process was investigated by studying the reactions, with deoxyguanosine, of the highly mutagenic model compound, 4-(carbethoxynitrosamino)-1-(3-pyridyl)-1-butanone, which is an analogue of NNK not requiring metabolic activation. A major adduct with deoxyguanosine was characterized as 2'-deoxy-N-(1-methyl-3-oxo-(3-pyridyl)-propyl)guanosine. Investigations of the formation of this and related adducts in vivo from NNK are continuing.

Epidemiological studies have established that the oral users of snuff (snuff-dippers) face a significantly higher risk for cancer of the oral cavity, especially cancer of the gums, than nontobacco users. It has been (5) established that snuff contains the carcinogen polonium-210 (0.2-1.2 pCi/g) and at least 12 carcinogenic N-nitrosamines. Of these, the tobacco-specific N-nitrosamines (TSNA) are the most carcinogenic and abundant ones, exceeding in concentrations by at least three orders of magnitude the upper limits set by the FDA for nitrosamines in consumer products. A study of snuff-dipping college students has demonstrated that the TSNA are extracted by saliva and that TSNA concentrations in saliva increase with length of habituation. Among the tobacco-specific nitrosamines, N'-nitrosonornicotine (NNN) and 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are derived from nicotine by nitrosation and are powerful carcinogens. They are not only organ-specific carcinogens which induce cancer of the nasal cavity, esophagus and lung in mice, rats, and hamsters, but administration of NNN and NNK by oral swabbing also leads to oral tumors in rats. The TSNA are metabolically activated by alpha-hydroxylation to active species. Upon metabolic activation NNK

is capable of forming O⁶-methylguanine, a promutagenic DNA adduct in vitro in both animal and human tissues and in vivo in animals. In a recent bioassay it was demonstrated that snuff, when repeatedly installed in a surgically created canal in the lower lip of rats induces benign and malignant oral tumors, although the yield of tumors was not significant. Work is continuing with this model for determining effects of smokeless tobacco components.

There are two studies in the Smoking and Health area that are supported by the contract mechanism. In one study (12) 36 male and 36 female smokers of cigarettes yielding between 14 and 17 mg of tar were recruited to assess the effects of switching to lower-yield cigarettes. Five commercial brands of cigarettes, with tar yields ranging from 15.4 mg down to 1.1 mg, were used in the experiment. It was found that the smokers consumed significantly more of the lower-yield brands and decreased the interval between puffs, but did not change in the duration or volume of the puff, or the number of puffs per cigarette. In spite of the increase in numbers of cigarettes smoked, there were significant decreases in both plasma cotinine and nicotine levels. This indicates that a reduced intake per cigarette more than offset the increase in daily cigarette consumption. No significant changes were observed in expired air carbon monoxide, possibly due to endogenous and exogenous confounding factors. Data are now available from a follow-up study (conducted by Franklin Institute under grant number R01 CA 38640-01, which expired last year) on the effect of switching brands of cigarettes. Two hundred three smokers who had participated in smoking-related studies 3-to-6 years earlier were retested to assess changes in their smoking practices. Those who had not changed brands in the interim had, on average, no change in plasma cotinine concentrations (reflecting exposure to nicotine) or in expired air CO, since the original testing. Those who had switched to lower yield brands, significantly reduced their daily cigarette consumption. The combination of lower yield and fewer cigarettes resulted in significantly reduced plasma cotinine concentrations, but only insignificant reductions in expired air CO. Smokers who had switched to higher yield cigarettes had significantly higher plasma cotinine concentrations and expired air CO. These results reflect long-term voluntary smoking practice changes and resulting physiological changes.

The second contract (13) serves as a resource for support in smoking studies by the NCI and scientific community. A concept for continuation of this effort was presented to the Division of Cancer Etiology Board of Scientific Counselors at the February 20-21, 1986 meeting. The concept was approved with the stipulation that work requested by the scientific community, and agreed by the Project Officer, be conducted on a "payback" basis. This procedure will be implemented. During the past 4 years, there has been an increasing awareness of a possible major health problem concerning the environmental air pollution from smoking tobacco products. Indoor air is contaminated by tobacco smoke constituents through direct emissions of lit cigarettes, cigars, and pipes during smoulder and by exhaled smoke from users. Cigarette smoke is of primary concern because cigarettes are consumed most commonly and in the greatest number. Sidestream smoke (SS), commonly defined as the smoke which issues from the products between puffs, is viewed as the most important emission. Most of the data currently available on sidestream smoke emissions has been generated by collecting the sidestream directly from the burning tip. The cigarette is enclosed in a container and smoked using standard puff conditions to collect mainstream smoke (MS), while simultaneously purging the container with air to collect sidestream smoke. Results are most commonly reported as mainstream deliveries and SS/MS ratios. Quantities of individual constituents released in the sidestream smoke typically vary by several orders of magnitude when calculated from SS/MS ratios and MS delivery ranges. Ambiguities

also exist in the phase distribution of various constituents. Sidestream nicotine, for example, has been reported as observed to reside primarily in the vapor phase while other studies indicate that it is primarily in the particulate phase, and still others report it distributed between the phases. Hydrogen cyanide has been reported as solely particulate phase and in other cases as preferentially vapor phase. A review of the tobacco literature and previous program experience suggests that sidestream deliveries are much more consistent than is suggested by results computed from SS/MS ratios. This is because MS deliveries are affected to a great extent by variables which only slightly affect SS deliveries. Conflicting results concerning the phase distribution of individual constituents and the sometimes poor agreement between laboratories for quantitative sidestream often appear to be related to smoke generation and collection systems used.

Fundamental research on cigarette combustion and smoke formation has shown that reductive, pyrolytic, and distillative processes contributed greatly to the quantities and nature of sidestream smoke emissions. Further research is required to develop validated sidestream cigarette smoke generation, sampling devices, and ultimately the determination of chemical characteristics of environmental tobacco smoke. Research studies in this area will continue to receive support.

SMOKING AND HEALTH
GRANTS ACTIVE DURING FY86

<u>Investigator/Institution/Grant Number</u>	<u>Title</u>
1. BENOWITZ, Neal L. Univ. of California (San Francisco) 2 R01 CA 32389-04	Nicotine and Tar Intake during Cigarette Smoking
2. CASTAGNOLI, Neal, Jr. Univ. of California (San Francisco) 5 R01 CA 35678-03	The Pharmacological Role of Nicotine
3. FOILES, Peter American Health Foundation 2 R01 CA 32391-04	Tobacco-Specific Nitrosamine: RIA for DNA-Adducts
4. HECHT, Stephen S. American Health Foundation 2 R01 CA 21393-10	Metabolism of the Carcinogen Nitrosonornicotine
5. HOFFMANN, Dietrich American Health Foundation 5 P01 CA 29580-05	Experimental Tobacco Carcinogenesis
6. HOFFMANN, Dietrich American Health Foundation 5 R01 CA 35607-03	Endogenous Formation of Nicotine Derived N-Nitrosamines
7. MARSHALL, Milton V. Southwest Foundation for Biomedical Res. 5 R01 CA 33069-03	Carcinogen Metabolism in the Cigarette Smoking
8. MC COY, George D. Case Western Reserve University 5 R01 CA 32126-03	Role of Ethanol in the Etiology of Head and Neck Cancer
9. POMERLEAU, Ovide F. University of Michigan at Ann Arbor 5 R01 CA 42730-02	Cholinergic/Beta-Endorphinergic Reinforcement of Smoking
10. SHODELL, Michael J. Cold Spring Harbor Laboratory 1 R13 CA 39347-01	Banbury Meeting on New Aspects of Tobacco Carcinogenesis
11. STITZER, Maxine L. Johns Hopkins University 5 R01 CA 37736-03	Tobacco Yield Changes: Behavioral and Biological Effect

SMOKING AND HEALTH
CONTRACTS ACTIVE DURING FY 86

<u>Investigator/Institute/Contract Number</u>	<u>Title</u>
12. LYNCH, Cornelius J. The Franklin Research Institute N01-CP-31047	Cigarette Smoke Yield and Smoker Compensation
13. GUERIN, Michael Department of Energy Y01-CP-30508	Collection, Separation, and Elucidation of the Components of Cigarette Smoke and Smoke Condensates

SUMMARY REPORT

CHEMICAL RESEARCH RESOURCES

The Chemical Research Resources program of the Branch endeavors to make available to the cancer research community those critical resources which are difficult or impossible for most investigators to obtain on their own, but which are necessary for the pursuit of studies on the chemical and physical aspects of carcinogenesis. Five resource contracts totalling \$1,252,825 million in FY 86 dollars presently comprise this program. There are no grants included. A major effort of this program has involved the synthesis and distribution of chemical carcinogens, derivatives, and metabolites for use as authentic research standards. Some of these compounds of major interest are available with ^{14}C or ^3H labeling.

The Research Resources program currently has four contractors who are involved in the synthesis of carcinogenic compounds and/or their metabolites. These contractors develop suitable routes for the unequivocal organic synthesis of compounds designated by the NCI project officer and then develop methods for production of adequate quantities of well-characterized compounds of high purity (generally greater than 98%). Compounds are analyzed by a meaningful combination of techniques to assess purity and confirm structure. These may include ultraviolet, fluorescence and/or infrared spectrometry, nuclear magnetic resonance, mass spectrometry, high pressure liquid chromatography, thin-layer chromatography, and elemental analysis.

During this report period a recompetition was held for the "Synthesis of Derivatives of Polynuclear Aromatic Hydrocarbons." Following the review of proposals submitted in response to the Request for Proposal (RFP), the two incumbents American Health Foundation and Chemsyn Science Laboratories (a subsidiary of Eagle Picher Industries) were selected for award. At the American Health Foundation (2) the current contract continues to work on the synthesis of quantities of key metabolites of benzo(b)fluoranthene (B(b)F), benzo(j)fluoranthene (B(j)F), and benzo(k)fluoranthene (B(k)F) for distribution to the research community through the NCI Chemical Carcinogen Reference Standard Repository. The benzofluoranthenes are among the most prevalent of the carcinogenic environmental polynuclear aromatic hydrocarbons (PAH), but in contrast to other hydrocarbons, such as benzo(a)pyrene, relatively little is known about the mechanism by which they cause cancer. Since many of these standards are now available, it is hoped that research will be stimulated in this area. During the past year, synthetic work has focused on B(b)F because of its relatively high carcinogenicity. All 12 possible phenolic metabolites of BbF have been synthesized, characterized and placed in the Repository. In addition, B(b)F-9,10-diol-11,12-epoxide, a potential ultimate carcinogen of B(b)F, has been prepared. These standards complement the three metabolic B(b)F dihydrodiols which were placed in the Repository earlier. Also, the potential quinone metabolites, B(b)F-9,10-dione, B(k)F-2,3-dione, and B(k)F-7,12-dione have been synthesized. The most recent additions to the Repository include 1-, 2-, and 3-methyl B(b)F, the first of the planned complete series of methylated benzofluoranthenes. The 3-methyl B(b)F has been shown to be exceptionally tumorigenic. The projected goals of this contract include the synthesis of standard characterized 3'-phosphates of polynuclear aromatic hydrocarbon-DNA adducts which will be valuable for a wide range of studies such as use in the Randerath ^{32}P -postlabeling assay.

The second successful bidder in the recompetition, Chemsyn Science Laboratories (9), is involved with the synthesis of NCI-selected, nonlabeled and labeled (^3H , ^{14}C) PAH derivatives other than the benzofluoranthenes. The parent compounds of interest for synthesis work include benz(a)anthracene, benzo(a)pyrene, benzo(e)pyrene, cyclopenta(c,d)pyrene, chrysene, dibenzanthracene and 3-methylcholanthrene. Derivatives of the following types are prepared as research trends dictate: phenols; quinones; epoxides; dihydrodiols; diolepoxides; alkyl and hydroxyalkyl substituted parent hydrocarbons; nitro-PAH derivatives; PAH-DNA adducts; and sulfate, glucuronide, and glutathione conjugates. The Chemsyn Science Laboratories maintains a Radiochemical Repository for the NCI under this contract. Quantities of isotopically labeled PAH metabolites are prepared and monitored for shipment to authorized recipients as directed by the NCI project officer. During the past 12 months 85 polynuclear aromatic hydrocarbon derivatives were synthesized, characterized, and shipped to the NCI Chemical Carcinogen Reference Standard Repository or, in the case of the isotopically labeled derivatives, placed in the Radiochemical Repository. This group included 45 new compounds. These derivatives have included ^{14}C - and ^3H -labeled racemic anti- and syn-dihydrodiol epoxides of benzo(a)pyrene (BP); K-region phenols, dihydrodiols, and ^3H -labeled derivatives of indeno(1,2,3-c,d)pyrene; non-K-region A-ring phenols, dihydrodiols, and the epoxide of 7,12-dimethylbenz(a)anthracene; methylpyrene derivatives; multifunctional derivatives of BP (e.g., 9-phenol-4,5-dihydrodiol); and re-synthesis of other labeled phenols, dihydrodiols and epoxides.

The Radiochemical Repository referred to above has a present inventory of 53 radiolabeled (^3H , ^{14}C) polynuclear aromatic hydrocarbon derivatives. A payback mechanism, whereby a portion of the synthesis and all of the handling and shipping costs for these compounds are recovered, has been in place since April 1, 1983. Since the initiation of payback, a total of 924 radiolabeled samples have been sent to investigators at a total billed cost of \$95,583.

A second contract (6) at Chemsyn Science Laboratories and a companion effort at SRI International (5) provide for the resynthesis of PAH derivatives as their main objective in order to maintain the inventory at the Repository. Once an unequivocal route has been developed and tested several times by the previously mentioned contractors, then Chemsyn Science Laboratories and SRI International provide the future resyntheses in order to maintain a continuing supply. Each contractor has specific parent PAH compounds for which responsibility is assigned for the preparation of derivatives. A second objective for these contractors is the syntheses of compounds from other chemical classes that are needed in the Repository: nitrosamines, aromatic amines, additional parent polynuclear aromatic hydrocarbons, aflatoxins, steroid derivatives, and physiologically active natural products, to name a few.

The efforts at the Chemsyn Science Laboratories resulted in the synthesis or resynthesis, purification, and characterization of 12 compounds. These are derivatives of benzo(a)pyrene, benzo(a)anthracene and cyclopenta(c,d)pyrene. Synthesis of other derivatives of these parent compounds is continuing with seven benzo(a)pyrene compounds and one cyclopenta(c,d)pyrene in progress at this time.

During this report period, the major work under the contract at SRI () has involved the synthesis and subsequent production of fecapentaene-12 and fecapentaene-14. This goal has been accomplished and over one gram of fecapentaene 12, and 150 mg of fecapentaene-14 have been shipped to the scientific community. The procedure for labeling fecapentaene has been under study for

sometime and a deuterium labeled form has been prepared. It is expected the labeled compound will be available before the end of 1986.

Another effort was assumed under this contract in June of 1985. For the past 5 years, under another contract, SRI has made available a number of labeled retinoids, in limited quantities, for research purposes. Most of these compounds were used in support of biochemical, metabolic, and pharmacologic investigations related to the chemoprevention of cancer. This activity continued under the existing contract. During the past year 19 shipments containing a total of 33.0 mCi. of radiolabeled retinoids were made to investigators in the United States, Sweden, and France. A total of \$7,500 has been collected from the payback activity. The development of new synthetic methodologies for the radiolabeled retinoids prepared under the predecessor contract resulted in ten technical articles and several presentations.

A recompetition for the operation of the Chemical Carcinogen Reference Standard Repository was carried out during the past year. After receipt and technical review of proposals a contract was awarded to Midwest Research Laboratories located in Kansas City, Missouri (1). All inventory, stock chemicals, and records were transferred from IITRI (the incumbent contractor) to Midwest Research Institute during November 1985 and the Repository operation resumed in December of 1985. All nonlabeled compounds (except for the fecapentaenes) prepared under the four synthesis contracts described previously are forwarded to the Chemical Carcinogen Reference Standard Repository. Other items of inventory are derived from surplus, reanalyzed chemicals that are tested by the National Toxicology Program (within the National Institute of Environmental Health Sciences) and surplus of other chemicals which are purchased commercially and reanalyzed. Most commercial purchases are made as a result of a need to obtain a given chemical for in vitro testing. The Repository participates in a program for the Office of the Director, Division of Cancer Etiology, in which selected chemicals are submitted as blind-coded samples for in vitro testing and subsequent evaluation as candidates for in vivo testing. During the past year, 673 shipments were made to the research community at large (this included shipments by IITRI (3) and Midwest Research Institute). These shipments contained custom packaged samples usually with 5 to 100 milligrams of material. Samples were furnished with analytical documentation and safety data sheets. General information on the handling and disposal of carcinogens has been provided in response to inquiries. This contract enables the NCI to provide compounds for pertinent experiments in chemical carcinogenesis which could not be carried out otherwise. Carcinogenesis research has been greatly stimulated by the availability of authentic reference standards and/or substrates. This can be attested to by the volume of published accounts of research citing the NCI Chemical Carcinogen and Radiochemical Repositories (IITRI and Chemsyn Science Laboratories) as the source of materials.

On April 1, 1983 the Chemical Research Resources program introduced a user's fee, or payback system, for samples distributed under the program. A price structure was developed which includes cost centers for the chemical cost, the handling/packaging cost and the shipping cost. Because of the great expense involved in developing a synthesis route for a new chemical, the NCI will still be significantly involved in the support of these contract efforts. The repository contractors bill the requestors and deduct the net income from their operating costs. The NCI then covers the balance of each month's operating cost to the contractors. The amount billed under the payback system, between April 1983 and April 1986, for unlabeled compounds was \$200,653 by IITRI and Midwest Research Laboratories combined.

RESEARCH RESOURCES

CONTRACTS ACTIVE DURING FY86

<u>Investigator/Institution/Contract Number</u>	<u>Title</u>
1. GRAVES, Steven Midwest Research Institute N01-CP-51012	Chemical Carcinogen Reference Standard Repository
2. HECHT, Stephen S. American Health Foundation N01-CP-15747	Synthesis of Derivatives of Polynuclear Aromatic Hydrocarbons
3. KEITH, James N. IIT Research Institute N01-CP-05612	Chemical Carcinogen Reference Standard Repository
4. PETRAZZOULO, Gary Technical Resources, Inc. N01-CM-57658	Working Group on Neoplasms and Related Disorders in Fishes
5. REIST, Elmer J. SRI International N01-CP-41028	Synthesis of Selected Chemical Carcinogens
6. RUEHLE, Paul H. Eagle Picher Industries, Inc. N01-CP-41001	Synthesis of Selected Chemical Carcinogen Standards
7. SHEALY, Y. Fulmer Southern Research Institute N01-CP-26009	Synthesis of Kilogram Amounts of Retinoids for Chemoprevention and Toxicity Studies
8. WILEY, James C. Eagle Picher Industries, Inc. N01-CP-05613	Synthesis of Derivatives of Poly- nuclear Aromatic Hydrocarbons
9. WILEY, James C. Eagle Picher Industries, Inc. N01-CP-61037	Synthesis of Derivatives of Poly- nuclear Aromatic Hydrocarbons

ANNUAL REPORT OF
LOW LEVEL RADIATION EFFECTS BRANCH
CHEMICAL AND PHYSICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1985 through September 30, 1986

The Low Level Radiation Effects Branch (LLREB), established in response to Public Law 95-622, plans, directs and administers a program consisting of grants and contracts investigating the means by which exposure to ionizing and non-ionizing radiations, particularly at low doses or dose rates, leads to molecular and cellular events and processes resulting in mutagenesis, cell transformation, and carcinogenesis, and the associated dose-effect relationships; directs and administers selected epidemiological studies investigating the effects of radiation exposure in humans; provides a broad spectrum of information, advice, and consultation to scientists and to institutional science management officials relative to the National Institutes of Health (NIH) and National Cancer Institute (NCI) funding and scientific review policies and procedures, preparation of grant applications, and choice of funding instruments; maintains contact with other Federal agencies and institutions and with the broader relevant scientific community to identify new and needed research in, and related to, the fields of radiation mechanisms and effects; provides NCI management with recommendations concerning funding needs, priorities, and strategies for the support of relevant research areas consistent with the current state of development of individual research elements and the promise of new initiatives; provides information, advice, and guidance to NCI management and staff on radiation-related issues; implements the mandates of Public Law 97-414, Section 7(a), and Public Law 98-542, Sections 7(a)(2) and 7(b); and represents the Department of Health and Human Services on the Science Panel of the Committee on Interagency Radiation Research and Policy Coordination, which is located within the Office of Science and Technology Policy, Office of the President.

The extramural activities of the Branch are accomplished through contractual agreements with universities and other Federal agencies, and through traditional individual research grants, program project grants, and conference grants with universities and research organizations. At present the Branch administers almost 100 research activities with an annual budget of over 13.6 million dollars. The program, although of insufficient size to justify sections, consists of two broad categories of research: mechanisms of radiation damage and repair, and radiation carcinogenesis. In addition, the NIH and the NCI have assigned to the Branch responsibility for the implementation of sections of two Public Laws addressing radiation-related issues emanating from Congressional policy concerns.

Section 7(a) of Public Law 97-414, the Orphan Drug Act, requires the Secretary to conduct scientific research and prepare analyses necessary to develop valid and credible (1) assessments of the risks of thyroid cancer that are associated with thyroid doses of Iodine-131; (2) methods to estimate the thyroid doses of Iodine-131 that are received by individuals from nuclear weapons fallout; and (3) assessments of the exposure to Iodine-131 that the American people received from the Nevada atmospheric nuclear bomb tests. A working committee consisting of relevant expertise within and outside of the government, including foreign

nationals, has been formed and is addressing these issues. The committee is organized into three task groups addressing the risk of thyroid cancer per unit dose of Iodine-131 to the thyroid, the dose of Iodine-131 to the thyroid per unit of exposure to Iodine-131, and the development and verification of models to estimate the exposure of the American people to Iodine-131 resulting from radioactive fallout associated with atmospheric nuclear weapons tests at the Nevada Test Site. Meetings of the full committee and of the several task groups have been held during this year. In addition, the required dose reassessments are being carried out via interagency agreements and with the assistance of staff expertise acquired for this purpose. An interim report to the Congress is in preparation and research needs are being identified. A final report will not be available for several years.

Public Law 98-542 requires the Director of the NIH to conduct a review of the reliability and accuracy of scientific and technical devices and techniques which may be useful in determining previous radiation exposure (e.g., among military personnel who participated in atmospheric nuclear weapons tests conducted by the United States or in the American occupation of Hiroshima or Nagasaki, Japan), including the availability of such devices and techniques, the categories of exposed individuals for whom the use of such devices and techniques may be appropriate, and the reliability and accuracy of dose estimates which may be derived from such devices and techniques. A review is being conducted, via an interagency agreement, by working groups to address the several relevant devices and techniques (e.g., whole body counting, bioassay studies, chromosomal alterations). A report to the Congress is anticipated early in 1987.

The Mechanisms of Radiation Damage and Repair program includes, but is not limited to, studies on molecular and cellular changes resulting from exposure to radiation, DNA damage and repair following radiation exposure, the hypermutability, mutagenesis, and malignant transformation of cells exposed to ionizing and nonionizing radiation, mutagenicity-carcinogenicity relationships following exposure to radiation, interspecies comparisons, and the interaction between cocarcinogens.

The Radiation Carcinogenesis program addresses the effects of exposure to radiation, including, for example, the role of oncogenes, studies of the sensitivity of the embryo or fetus to ionizing radiation, the effect of dose rate and linear energy transfer on radiation-induced effects, dose-effect relationships, interspecies comparisons, cocarcinogens, the incidence of selected diseases as they may relate to exposure from radioactive fallout, and synthesis of radiobiological data in the assessment of risk and the establishment of appropriate radiation protection practices.

The LLREB contributed to the support of two conferences held during the year addressing topics of relevance to the Branch: "Radiation Carcinogenesis and Associated Dosimetry" and "Radiation and Health."

TABLE I
LOW LEVEL RADIATION EFFECTS BRANCH
(Extramural Activities - FY 1986 - Estimated)

	No. of Contracts/Grants	\$ (Millions)
Research Contracts	9	1.90
Research Grants	90	11.72
Traditional Project Grants (86 grants; \$10.63 million)		
Conference Grants (3 grants; \$0.02 million)		
Program Project Grants (1 grant; \$1.07 million)		
	—	—
TOTAL	99	13.62

TABLE II
LOW LEVEL RADIATION EFFECTS BRANCH
(Contracts and Grants Active During FY 1986)

	FY 86 (Estimated)			
	CONTRACTS		GRANTS	
	No. of Contracts	\$ (Millions)	No. of Grants	\$ (Millions)
Radiation Mechanisms and Carcinogenesis	6	1.13	87	11.70
Office of the Chief	<u>3</u>	<u>0.77</u>	<u>3</u>	<u>0.02</u>
TOTAL	9	1.90	90	11.72

Research activities are concerned with a wide variety of radiation effects including mechanisms of damage and repair of DNA by ionizing and nonionizing radiation, and radiation carcinogenesis. The majority of the 90 grants (66) support investigations relating to mechanisms of radiation damage and repair of cellular DNA, 42 of which investigate the effects of exposure to ionizing radiation and 24 of which study the consequences of exposure to ultraviolet (UV) radiation, visible light and ultrasound. Nineteen grants and eight contracts fund studies in radiation carcinogenesis, and research addressing radiation risks and the compilation and assessment of information is supported by five grants and one contract.

Mechanisms of Radiation DNA Damage and Repair: The LLREB provides substantial support for basic research related to mechanisms of mutagenesis and malignant transformation by radiation. This information is essential for providing not only insight into the mechanisms of carcinogenesis, but also the rationale for the carcinogenic effects seen following exposure to radiation. Such studies contribute to the evaluation of risks to individuals and to populations and thereby assist in providing a data base upon which radiation standards might be developed.

A. Ionizing Radiation: In the past few years evidence has accumulated that DNA repair is far more complex than anticipated and that the classical nucleotide excision pathway is integrated with other equally important mechanisms to maintain the integrity of DNA. Previous investigations studying the preferential formation of DNA-protein cross-links and single strand DNA breaks in areas of the genome containing transcriptionally active genes found that after x-irradiation two nonhistone proteins which are minor components of the nuclear matrix become covalently bound to the DNA. During the repair process the protein linked to DNA in regions of transcriptionally active areas is removed, following which other areas of the genome are bound. Now an investigator has determined that there are about 6000 DNA-protein cross-links in the normal cell and that for each Gy of x-irradiation an additional 150 cross-links are induced. These x-ray-induced cross-links are preferentially formed between actively transcribing DNA and protein of the nuclear matrix. This was demonstrated by showing that the mouse globin gene is hypersensitive to formation of radiation-induced DNA-protein cross-links only when it is in the matrix-associated transcribing mode of stimulated erythroleukemia cells but not in the unstimulated mode of erythroleukemia cells or in fibroblasts. Similar experiments showed that a heat-shock gene also was hypersensitive to DNase I digestion in the transcribing mode but not sensitive to digestion in the latent, "turned off," mode. These results underscore the complexity of cellular responses and show that the DNA target genome is not equally sensitive to x-ray throughout its length. Further, sensitivities to radiation not only change during the cell cycle, but they change according to whether or not the gene is activated or suppressed.

Cells damaged by radiation can repair at least some of the damage and thereby increase their probability of survival. However, in a complex organism simple survival of individual cells may be more detrimental than cell death if any of the surviving cells are transformed to cancer cells. Thus, an important concern for the LLREB is to determine to what extent, and with what degree of fidelity, human cells can repair following exposure to varying doses and types of radiation (e.g., high vs. low LET). In this regard the concept of "potentially lethal damage" (PLD) has stimulated much work. Although it has for many years been

known that proliferating cells and tissues exhibited a greater degree of radio-sensitivity than did nonproliferating cells and tissue, the concept arose directly from the finding that cells in culture which are rapidly proliferating cannot tolerate as much radiation as cells which are in the resting phase. This suggested the idea that in the resting phase there is more time for repair, while the proliferating phase cells tend to continue cycling and are unable to repair to the same extent as resting cells. The result is that survival is much greater in resting cells. However, two studies cited last year suggested that adequate nutrient was an important factor for interpretation of these experiments. This year an investigator studying PLD repair in V79 cells focused attention on a serious problem with regard to the stationary or plateau phase of these experiments. After confirming other research results, it was determined that the best explanation for increased survival after "holding" experiments was that apparently proliferation of the cells during "holding," not DNA repair, best accounted for the observed increase in survival.

The mechanism(s) of oxygen enhancement of x-irradiation damage has been intensively studied and it is widely held that superoxide anion radicals $\cdot O_2^-$ and/or perhydroxyl radical, $\cdot O_2H$, have significant damaging roles. However, technical problems complicate the conclusions and raise questions as to the actual identity of the damaging agents. Recent investigations indicate that in at least one system the above agents are likely not involved. When two appropriate scavengers are chosen based on reactivity, toxicity, and cell permeability considerations, it was determined that the amount of sensitization depends simply on the O_2 concentration present; no protection was afforded when $\cdot O_2^-$ and $\cdot O_2H$ formation was blocked. The conclusion reached, that radiation sensitization by O_2 in V79 cells does not require the production of superoxide or perhydroxyl radical, suggests that function of the enzyme superoxide dismutase may not be primarily protective.

It has been generally accepted that the great majority of biological effects of x-irradiation are a result of DNA strand breaks, mostly double strand breaks, and deletions or rearrangements in DNA. Early hints that perhaps point mutations were important arose from investigations on oncogene activation by x-irradiation in which the activated ras oncogene was shown to be mutated in one of its characteristic locations. An investigator has now determined by restriction fragment length polymorphisms at specific genetic loci that a sizable fraction of x-ray-induced mutations in human cells (TK6) are point mutations (approx. 40%), while the remainder (approx. 60%) are large-scale genetic changes. Additional results which add support to the above are findings by another investigator that more than 15% of the lethal events in x-irradiated PM2 bacteriophage result from saturation of the thymine ring structure and production of alkali-labile lesions; further, as much as 55% of the lethal lesions seem to be other types of base damage as yet undefined. These results are significant because they emphasize the extensive overlap of biological effects and cell responses to x-irradiation, ultraviolet light, and chemical damage.

Additional significant results include: (1) the finding that two kinds of DNA 3'-end lesions are induced by x-irradiation: a 3'-phosphoryl and a 3'-phosphoglycolate. The lesions are caused largely by $\cdot OH$ since 250 M 2-mercaptoethanol completely abolishes their formation. This is significant because it tells us the specific type of enzymatic activity that is necessary to initiate repair, i.e., hydrolysis to 3'-OH ends. (2) Cells (E. coli) devoid of glutathione are not hypersensitive to agents that cause damage through oxygen radicals, and, in

fact, they even show a slightly elevated resistance. This is significant because there is abundant indirect evidence suggesting that glutathione is an essential component of at least one oxidation protection mechanism, the glutathione-peroxidase system. (3) A repair enzyme, gamma-endonuclease from *M. luteus*, was purified to homogeneity. It consists of a single polypeptide that has two distinct activities: a glycosylase which releases x-ray-altered bases from DNA and a Mg^{++} -independent apurinic endonuclease. This finding extends the extraordinary complexity of DNA repair structures from the "repairosome" aggregate down to the individual polypeptide. (4) DNA recombination events have long been reported to be associated with the cellular response to DNA damage, especially ionizing radiation damage. Studies in drosophila DNA repair mutants that are defective in P-element mobility (transposon mobility) are especially sensitive to x-irradiation damage. This sensitivity supports the important conclusion that the genetic damage common to ionizing radiation and P-element mobility is the induction of chromosome breaks and rearrangements. This provides strong support for the concept that recombination events are important processes in DNA repair. Further, other studies with extracts of mouse cells indicate that the cellular components involved in homologous recombination are not induced by x-irradiation and appear to be constitutive.

A series of experiments using high-LET radiation with repair-deficient and repair-proficient cells demonstrated that cell DNA repair processes must be considered when formulating theories and models of cell radiosensitivity. It was shown that repair competence in cells is associated with a change in kinetics of survival, and the matched repair-deficient controls rule out possible objections as to differences in cell type, species, time in culture, etc. Other findings indicate that low dose rates of high-LET radiation may be a tumor-promoting agent and warrant further investigation. Recently an investigator discovered that in human and rodent cells mutations induced by fission-spectrum neutrons are dose-rate dependent: low-dose-rate exposures (0.5 rad/min) induced significantly more mutants than high-dose-rate exposures (20 rad/min). However, as had been previously observed, cell killing is independent of neutron exposure rate. Also, neutrons produce far fewer single strand breaks than gamma rays and they are rapidly repaired, so single strand breaks themselves are probably not responsible for the cell killing caused by high-LET irradiation. These studies are of potential importance in high-LET radiation therapy and in the prediction of the effects on low doses of high-LET radiation delivered at low dose rates.

Another investigation which is relevant to the question of adding a repair term to the theory of cell sensitivity to radiation suggests that low-LET radiation may not be either linear or involve a threshold, the two generally favored theories. This investigator believes that using conventional methods, mutation rates are underestimated because of the role of killing by lethal mutation. Methods which permit separate evaluation of localized mutations, deletions, translocations, and nondisjunctional processes indicate that the actual dose response curve exhibits a downward concavity so that the mutational efficiency is maximal at low doses. Development of a valid model for genetic damage and organism (cell) response have important implications for human health.

B. Ultraviolet Radiation: Ultraviolet radiation is a ubiquitous carcinogenic agent in the environment. However, the LLREB is not only interested in its direct effect, but also in its plausible role as a cocarcinogen and the relationship of its repair pathway to the repair pathways for ionizing radiation. Sorting

out and documenting the various biochemical pathways and properties has historically provided new methods and biological reagents for further advances, both practical and conceptual.

The transfection technique of DNA information transfer has become a powerful method of introducing specific genes into mammalian cells after appropriate manipulation by recombinant DNA methods. However, most human cells have proven refractory to integration of the DNA into a stable state of expression. Last year it had been discovered that UV irradiation of chimeric plasmids such as pSV2-gpt results in a remarkable enhancement in the yield of gpt⁺ transformants when the plasmids are transfected into human cells by the calcium phosphate technique. Further, only "bulky" lesions, including pyrimidine dimers and psoralen adducts, cause this enhancement which can be as much as 20-fold. This investigator has now extended the work to show that pyrimidine dimers in the DNA sequences flanking the transforming genes in shuttle vectors are responsible for the marked transformation enhancement of human cells. Further, the pyrimidine dimers are the predominant lesion involved and the average number of copies of the gene integrated into the genome of the transformant is not affected by the damage. This investigator has also now developed techniques to measure specific DNA damage and its repair in a defined nucleotide sequence at the single-copy level in the mammalian genome. Using these techniques it has been discovered that the essential dihydrofolate reductase gene is proficiently repaired in normal human fibroblasts and in Chinese hamster ovary cells (CHO) with overall low repair ability, but not in cells from individuals with the hereditary disease, xeroderma pigmentosum (complement group C), which have low repair levels like CHO cells. These extraordinary experiments show that cellular survival correlates with efficient repair in an essential gene rather than with overall repair levels and resolves a long-standing anomaly concerning the relationship between DNA repair capacity and survival in rodents and human cells in culture. In addition to the usefulness of this discovery to manipulate DNA in human cells in culture, these results further emphasize the need to better understand the relationships which exist between DNA repair, replication, integration, and expression and to recognize the interdependence of these processes and their response to UV radiation.

By far most work concerning biological effects and cell responses to ultraviolet light focuses on DNA. Recent studies by an investigator suggest that biological effects of RNA lesions, not just DNA lesions, should be seriously considered. It was found that in RNA and DNA model compounds, photolesions in RNA have a much greater destabilizing effect on the RNA helix than on the DNA helix. Also, when positional effects of the lesion were studied, it was found that conformational perturbations were much larger in the 5'-direction than in the 3'-direction. These results, taken together with results from other investigations studying the effects of ultraviolet light on small molecular weight ribonucleoproteins in cells and the well-documented importance of precise RNA conformation for tRNA and splicing RNA, suggest this may be the beginning of a fruitful area of molecular biochemistry relating to the restoration and maintenance of the cell genome and metabolic integrity.

The use of simple organisms as models for more complex ones is a time-honored research technique. Last year an investigator discovered a postreplication repair pathway in *E. coli* which joins double strand DNA breaks that arise at unrepaired DNA daughter-strand gaps. Daughter-strand gaps occur when the DNA polymerase replication activity is interrupted because of a "bulky" adduct on

the template strand; polymerase activity continues after the adduct which leaves a small gap in the daughter-strand DNA opposite the adduct. This investigator has now discovered the same repair pathway in human cells. The significance of this is that double strand DNA breaks are believed to be among the most detrimental lesions, and the cellular response is crucial to the complex organism. For example, if their repair is error-prone or otherwise mutagenic this could significantly increase the probability of neoplastic transformation, the risk of which needs to be balanced with the risk of isolated cell death in the complex organism.

Radiation Carcinogenesis: The transformation of normal cells to malignant cells by ionizing and ultraviolet radiation and their subsequent expression as cancer is an undisputed fact. The objectives of research in this area within the LLREB program are to establish dose-effect relationships, including factors of dose rate and type of radiation; to determine whether there is a level of exposure to these agents which might be considered "safe"; to explore possibilities for intervening or ameliorating detrimental levels of exposure; and to expand the data base from which risk estimates are derived.

The risk of developing thyroid cancer following therapeutic radiation exposures is being studied in over 5,000 patients with a history of external irradiation to the head and neck for benign conditions during childhood. Several decades after exposure these patients are experiencing an increased risk for thyroid tumors. The clinical course of radiation-induced thyroid cancer seems to be the same as for nonradiation thyroid cancers observed clinically. Measurement of serum thyroglobulin appears to be an indicator of a developing tumor, with patients who had an increasing level of serum thyroglobulin being more likely to develop thyroid tumors.

The incidence of leukemia and thyroid disease in Utah is being assessed in relation to fallout from the Nevada Test Site between 1951 and 1962. Over 85% of more than 5,000 exposed and unexposed children identified and examined for thyroid disease in the 1960s have been located, and thyroid reexaminations of these persons, which constitute a "thyroid cohort" study, are in progress. Dose reassessments of subjects both of this cohort study and of a leukemia case-control study are being made.

The question of whether or not there are tumor-specific antigens and, if so, to what extent, is an extremely important question in cancer research because of the potential for cancer detection and treatment if they do exist to a significant extent. To test the tumor-specific antigen concept (that specific antigens are always associated with tumors and that they might be characteristic), an investigator determined whether UV radiation-associated antigens could be induced in poorly antigenic tumor cells by UV irradiation even though the cells were from a spontaneous tumor and not derived from a UV-induced tumor. It was found that the UV exposure induced antigens in the nonantigenic fibrosarcoma tumor cells and that these antigens were indistinguishable from antigens produced in vivo in mice exposed repeatedly to UV radiation. Another investigator, studying oncofetal antigens which are known to be formed on human and rodent carcinomas and sarcomas, has now demonstrated their presence on radiation-promoted myeloid leukemias and thymomas of mice and man. (Oncofetal antigens are proteins expressed in adults only under pathological conditions.) These results are essentially in agreement with the idea of exploitable tumor-specific antigens.

Two studies are investigating ways of ameliorating potential consequences resulting from exposure to radiation in particular situations. One of the studies is concerned with the possibility that a pregnant woman accidentally becomes contaminated with plutonium or transplutonium radionuclides. It was found that if fetal mice were exposed to ^{241}Am or ^{237}Pu from their mother, chelation therapy with Zn-DTPA would reduce the radioactive burdens of both the fetuses and the mother significantly. Further work will be needed to determine the degree of toxicity, if any, caused by the chelator, and the associated margin of cancer reduction in the offspring. The other study suggests that hormone treatment may be useful for preventing the occurrence of secondary cancers induced by radiation therapy. This investigator has observed that some hormones such as testosterone, dihydrotestosterone, and cortisol have the ability to suppress radiation-induced transformation in vitro, while other hormones such as $17\text{-}\beta\text{-estradiol}$, prednisone, and cortisone enhance radiation-induced transformation. These results suggest that the choice of hormones when combining radiation therapy with hormone treatment may be important in the level of risk for radiation-induced secondary cancers.

It is important to clarify the various biological and dosimetric factors which influence the expression of radiation effects (e.g., the strain/species of animal, the type of radiation, the meaning and relevance of "dose" at the molecular and cellular level, the significance of dose rate). Toward this end, several studies are being pursued. The relationship between the natural incidence of cancer and the susceptibility to radiation-induced cancers is under investigation with several different strains of mice. The basic question of whether radiation will be leukemogenic after doses that do not kill or perturb hematopoietic stem cells is being addressed in another study; single doses of 10 or 20 rad have not produced a detectable reduction in the number of stem cells, whereas a detectable decrease is evident after exposure to 40 rad. One study of the relationship between fission neutron dose and incidence of myeloid leukemia in mice has been completed; over the dose range 0-80 rad (lowest dose = 5 rad), the leukemia incidence was linear and the frequency of chromosome aberrations was also linear. Information such as this continues to contribute data upon which to determine the appropriate model for describing the effects of radiation on animals at relatively low doses.

Information that would contribute to resolving the question of whether or not it is valid to extrapolate radiation effects from species to species, at least within mammals, may emerge from an extensive study with dogs. This investigation is measuring the late effects of protracted whole-body gamma irradiation on beagles at four total doses (450-3000 rad) at four dose rates (3.8-26.3 rad/day; 22 hr/day). Preliminary results indicate: (a) no evidence for a dose rate effect, (b) death from tumors is proportional to total dose, (c) time of fatal tumor onset is shortened by increased total dose, and (d) increased total dose increases the number of nonfatal tumors. These results, when compared to similar ongoing studies on rodents, are expected to provide information regarding the similarity or differences in the radiation responses between rodents and dogs, and thereby may contribute to the data base upon which extrapolation to man may be made.

Evidence is accumulating that cocarcinogenesis is important to more fully understand the carcinogenesis process. It now appears that ultraviolet light as well as chemicals may be a cancer-promoting agent. Investigators have recently shown that mouse skin cells can be "initiated" by x-irradiation, with tumors subsequently induced in these animals by ultraviolet light promotion. Particularly significant

is the fact that after 16 fractions of x-rays (250 R, 2 x wk), ultraviolet light exposure (280-400 nm sunlamp) resulted in an incidence of more than 60% of squamous cell carcinomas under conditions where neither agent alone produced any tumors. Considering that x-rays plus the potent chemical 12-O-tetradecanoyl-phorbol-13-acetate (TPA) resulted in an incidence of about 80% under the same conditions, it is likely that sunlight is also a potent promoter. These results are important when considering both therapeutic doses of x-irradiation and the risks associated with such doses followed by exposure to sunlight.

There is concern that the irradiation of a developing embryo and fetus at low levels of exposure may have long-term detrimental effects not apparent in the early months after birth. Although not yet complete, studies are investigating the sensitivity of the embryo and fetus to ionizing radiation as a function of stage of development. Current data from a study with dogs indicate a statistically significant increase in tumor incidence and mortality during the first 4 years of life in perinatally irradiated dogs, as compared to unirradiated controls, which suggests an increased risk for neoplasia after perinatal irradiation. This is consistent with the findings from an earlier survey of malignant disease in childhood which suggested there was a relationship between prenatal exposure to diagnostic irradiation and subsequent development of leukemia and other cancers in children during the first 10 years of life. Preliminary studies have demonstrated significant radiation-induced thymic epithelial cell injury in dogs after prenatal exposures which could lead to immunoregulatory or immunodeficiency disorders. Such disorders have been associated with an increased prevalence of lymphoid neoplasia. Other investigators working with rodents in this area have focused on immunosuppressive effects of prenatal irradiation, late immunological effects of prenatal iodine-131 exposure, cocarcinogenic effects when prenatal exposures are followed postnatally with urethan treatment, and tumor initiation potential of in utero exposures when followed by treatments with the tumor promoter phorbol myristate acetate (PMA). Preliminary data from mice appear to indicate that prenatal irradiation decreases the tumor response from urethan after PMA promotion; additional data will determine if this finding is repeatable.

Though it appears unlikely that oncogenes are involved in the induction or initiation of malignant transformation, there is evidence indicating a role for the selection and/or progression of malignant cells. This possibility again raises the question of whether low levels of radiation exposure may, in fact, enhance the expression of these genes, thus increasing both cancer incidence and lethality. The evidence to date is mixed and interpretation is controversial. Investigations comparing radiation and chemical transformation and its correlation with oncogene activation found that x-ray-induced mouse mammary tumors did not exhibit detectable oncogene activation (3T3 transformation). On the other hand, the same investigator found that each DMBA-induced mammary tumor had the H-ras gene activated. The activation was due to a mutation in the 61st codon. Another investigator tested about 30 human melanomas in the metastatic and vertical growth phase. None of the DNAs transformed NIH 3T3 cells; however, each of the tumor DNAs induced anchorage independence in normal human cells which subsequently exhibited karyotypic abnormalities. A third investigator has preliminary evidence that fission spectrum neutrons, x-rays, and ultraviolet radiations each may activate different oncogenes in C3H10T-1/2 cells. Clearly, more research is needed to clarify the role(s) of oncogenes in cancer.

The LLREB also provides grant support to several national and international advisory bodies which analyze and disseminate information concerning, and provide

guidance on matters pertaining to, occupational and public radiation protection issues: the National Council on Radiation Protection and Measurements (NCRP), the International Commission on Radiation Units and Measurements (ICRU), and the International Commission on Radiological Protection (ICRP). During the year the ICRP produced three publications, including reports on quantitative bases for developing a unified index of harm, radiation protection principles for the disposal of solid radioactive waste, and radiation protection of workers in mines. One report has come from the ICRU on the quality factor in radiation protection. The NCRP has published six reports during FY 86, including one on the general concepts for the dosimetry of internally deposited radionuclides and another on the biological effects and exposure criteria for radiofrequency electromagnetic fields.

LOW LEVEL RADIATION EFFECTS

GRANTS ACTIVE DURING FY86

<u>Investigator/Institution/Grant Number</u>	<u>Project Title</u>
1. ALDERFER, James L. New York State Department of Health 5 R01 CA 39027-02	Effects of Light on Nucleic Acids
2. ANANTHASWAMY, Honnavara N. University of Texas System Cancer Center 2 R23 CA 40454-02	Relationship Between UV-Associated Antigens and Transformation
3. BALCER-KUBICZEK, Elizabeth K. University of Maryland at Baltimore 2 R23 CA 32729-04	Oncogenesis from Low-Dose-Rate Irradiation
4. BALCER-KUBICZEK, Elizabeth K. University of Maryland at Baltimore 1 R01 CA 42820-01	Mechanism of Microwave Carcinogenesis in Vitro
5. BARROWS, Louis R. George Washington University 1 R01 CA 41561-01	Repair of X-Ray-Induced DNA Damage: Genetic Basis
6. BASES, Robert E. Yeshiva University 5 R01 CA 36492-03	X-Ray Damage and Repair of Primate Cell A DNA Sequences
7. BEDFORD, Joel S. Colorado State University 5 R01 CA 18023-12	Dose and Time Factors in Cellular Radiosensitivity
8. BENJAMIN, Stephen A. Colorado State University 5 R01 CA 36456-02	Prenatal Thymic Radiation Injury and Immune Development
9. BERNHARD, William A. University of Rochester 5 R01 CA 32546-11	Solid State Radiation Chemistry of Nucleic Acid Bases
10. BOX, Harold C. Roswell Park Memorial Institute 5 R01 CA 25027-18	Transfer Mechanisms in Irradiated Biological Systems
11. BRENT, Thomas P. St. Jude Children's Research Hospital 5 R01 CA 14799-12	Enzymes and Reactions for Repair of DNA in Human Cells
12. CARDIFF, Robert D. University of California, Davis 2 R01 CA 36493-03	Radiation Activation of Oncogenes

13. CLAYCAMP, Gregg H.
University of Kansas
5 R01 CA 35380-03
Radiation Biochemistry of DNA
Base Damage
14. CLAYCAMP, Gregg H.
University of Iowa
7 R01 CA 43324-01
Radiation Biochemistry of DNA
Base Damage
15. CLIFTON, Kelly H.
University of Wisconsin Madison
5 R01 CA 13881-14
Radiation In Vitro Mammary
Neoplasia
16. COGGIN, Joseph H., Jr.
University of South Alabama
5 R01 CA 39698-02
Role of Oncofetal Antigens in
Radiation Carcinogenesis
17. COOPER, Priscilla K.
University of California, Berkeley
2 R01 CA 32986-04
Inducible Responses to Carcinogenic
DNA Damage
18. DEMPSEY, Bruce
Harvard University
5 R01 CA 37831-02
Oxidative DNA Damage: Repair
and Cellular Responses
19. DOETSCH, Paul W.
Emory University
1 R01 CA 42607-01
Repair of Oxidative and Radiation-
Induced DNA Damage in Human Cells
20. ELKIND, Mortimer M.
Colorado State University
5 R01 CA 33701-04
Cell Radiation Response at
Low Dose Rates
21. ELKIND, Mortimer M.
Colorado State University
1 R01 CA 41483-01
Radiation Transformation and Its
Modulation by Chemicals
22. ESSIGMANN, John M.
Massachusetts Inst. of Technology
2 R01 CA 33821-04
Genetic Effects of Ionizing
Radiation
23. EVANS, Helen H.
Case Western Reserve University
2 R01 CA 15901-12
Mutants and Altered Radioresponse
to Cells and Tumors
24. EVANS, Helen H.
Case Western Reserve University
5 R01 CA 23427-06
Radiation-Induced Mutagenesis
and Carcinogenesis
25. EWING, David
Hahnemann Univ. School of Medicine
5 R01 CA 28932-05
Lethal Damage from O₂ and OH
in Irradiated Cells
26. EWING, David
Hahnemann Univ. School of Medicine
5 R01 CA30921-02
Mechanisms of Damage in Irradiated
Cells

- | | |
|---|---|
| 27. GARTE, Seymour J.
New York Univ. Medical Center
1 R01 CA 43199-01 | Oncogene Activation in
Radiation Carcinogenesis |
| 28. GRIFFITHS, T. Daniel
Northern Illinois University
5 R01 CA 32579-05 | DNA Replication after Insult
with UV |
| 29. GRIGGS, Henry G.
John Brown University
5 R01 CA 18809-10 | Ultraviolet and Ionizing
Radiation Damage |
| 30. GUERNSEY, Duane L.
University of Iowa
2 R01 CA 36483-03 | X-Irradiation-Induced Oncogene
in Mouse Embryo Cells |
| 31. HALL, Eric J.
Columbia University
5 P01 CA 12536-15 | Effects of Small Doses of
Ionizing Radiation |
| 32. HALL, Eric J.
Columbia University
5 R01 CA 37967-03 | Oncogenic Transformation and High
LET Radiations |
| 33. HALL, Eric J.
Columbia University
1 R13 CA 40312-01 | Conference: Cell Transformation
in Radiobiology |
| 34. HANAWALT, Philip C.
Stanford University
5 R01 CA 35744-03 | Molecular Basis of DNA Repair
Deficiency in Xeroderma
Pigmentosum |
| 35. HARRISON, George H.
University of Maryland
5 R01 CA 40223-02 | Ultrasound and Malignant
Transformation In Vitro |
| 36. HENNER, William D.
Dana-Farber Cancer Institute
5 R01 CA 35767-03 | Ionizing Radiation-Induced DNA
Damage and Repair |
| 37. HILL, Colin
Univ. of Chicago
1 R01 CA 40496-01 | Neutron Energy: Dose Protraction
Effect on Transformation |
| 38. HILL, Colin
Univ. of Southern California
7 R01 CA 42808-01 | Neutron Energy: Dose Protraction
Effect on Transformation |
| 39. HUBBELL, Howard R.
Hahnemann University
5 R01 CA 37020-02 | Oncogenes in Chronic Myelogenous
Leukemia |
| 40. HUBERMAN, E.
University of Chicago
2 R01 CA 33974-04 | Mutation-Transformation: Neutron
Damage and Repair |

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| 41. | HUMPHREY, Ronald M.
Univ. of Texas System Cancer Center
5 R01 CA 04484-28 | DNA Repair and Recovery in
the Mammalian Cell Cycle |
| 42. | HUMPHREY, Ronald M.
Univ. of Texas System Cancer Center
2 R01 CA 24540-07 | The Importance of DNA Damage
and Repair for Cell Survival |
| 43. | ILIAKIS, George
Thomas Jefferson University
5 R01 CA 42026-02 | Has Cellular Repair (PLD,SLD)
Common Molecular Base? |
| 44. | KENNEDY, Ann R.
Harvard University
2 R01 CA 34680-04 | Hormones, Radiation, and
Malignant Transformation |
| 45. | KOPELOVICH, Levy
Friends Medical Science Research Center
5 R01 CA 39081-02 | Effects of Gamma Rays and Neutrons
on Human Mutant Cells |
| 46. | LANGE, Christopher S.
Downstate Medical Center
5 R01 CA 39045-02 | Radiosensitivity Prognosis Based
on DNA Repair Assay |
| 47. | LAUGHLIN, John
Memorial Sloan-Kettering Cancer Center
1 R13 CA 43355-01 | Conference on Radiation
Carcinogenesis and Dosimetry |
| 48. | LAWRENCE, David A.
Albany Medical College
5 R01 CA 35889-03 | Radiation Immunobiology of
Carcinogenesis |
| 49. | LEONE, Fred C.
American Statistical Association
1 R13 CA 41828-01 | ASA Conference on Radiation and
Health |
| 50. | LETT, John T.
Colorado State University
5 R01 CA 10714-16 | Repair of Radiation Damage
to Cellular DNA |
| 51. | LITTLE, John B.
Harvard University
5 R01 CA 11751-17 | Effects of Radiation on
Stationary Cells |
| 52. | LITTLE, John B.
Harvard University
2 R01 CA 34037-04 | Radiation Mutagenesis in Human
Cells |
| 53. | MAHLUM, D. Dennis
Pacific Northwest Laboratories
5 R01 CA 35912-03 | Prenatal Irradiation and Postnatal
Tumor Response |
| 54. | MAYS, Charles W.
University of Utah
5 R01 CA 28314-07 | Reducing Cancer Risk by
Radionuclide Chelation |

55. MERUELO, Daniel
New York University
5 R01 CA 35482-03
Cloning & Study of a Major Gene
Involved in Oncogenesis
56. NAIRN, Rodney S.
Univ. of Texas System Cancer Center
5 R01 CA 36361-03
Repair and Recombination in
Radiation Sensitive Cells
57. NELSON, William H.
Georgia State University
1 R01 CA 36810-01
Radiation Chemistry of Purines
in the Solid State
58. NORDLUND, Thomas M.
Univ. of Rochester Medical Center
1 R01 CA 41368-01
DNA Damage Studied by Ultrafast
Spectroscopy
59. OLEINICK, Nancy L.
Case Western Reserve University
5 R01 CA 15378-13
Radiation-Induced Modifications
in Protein Synthesis
60. PEAK, Meyrick J.
University of Chicago
5 R01 CA 34492-03
Solar UV Damage in Human Cells
61. PEAK, Meyrick J.
University of Chicago
5 R01 CA 37848-03
Biological Effects of Solar-UV-
Generated Oxygen Species
62. PETROVICH, Zbigniew
University of Southern California
7 R01 CA 38370-01
UV-X-Ray Interaction: Mutation
and Transformation
63. PIEPKORN, Michael W.
University of Utah
1 R23 CA 41591-01
Glycosaminoglycans of Skin
Tumors
64. PRAKASH, Satya
University of Rochester
1 R01 CA 41261-01
Repair of UV-Irradiated DNA:
Excision Genes of Yeast
65. RAMANATHAN, Brinda
Washington State University
1 R23 CA 43079-01
Nuclear Protein Modifications in
UV Damaged Human Cells
66. REDPATH, John L.
University of California, Irvine
5 R01 CA 27561-06
Photoreactivation of X-Ray-
Induced Damage in E. coli
67. REDPATH, John L.
University of California, Irvine
1 R01 CA 39312-01
Radiobiological Studies of Human
Hybrid Cell Lines
68. ROSSI, Harald H.
Columbia University
5 R01 CA 15307-13
Cell Irradiations with
Molecular Ions

69. SCHNEIDER, Arthur B.
Michael Reese Hospital & Med. Ctr.
5 R01 CA 21518-10
Radiation-Induced Thyroid
Cancer
70. SCOTT, Walter A.
University of Miami
5 R01 CA 35244-03
Radiation-Induced Genetic
Alterations in Mammalian Cells
71. SILVERSTONE, Allen E.
State University of New York
5 R01 CA 42128-02
In Vitro Culture of Radiation-
Induced Preleukemic Cells
72. SINCLAIR, Warren K.
National Council on Radiation Protection
2 R01 CA 18001-20
Radiation Protection and
Measurements
73. SMITH, Kendric C.
Stanford University
5 R01 CA 02896-30
Repair of Radiation-Induced
Lesions in DNA
74. SMITH, Kendric C.
Stanford University
5 R01 CA 06437-25
Molecular Basis of Radiation
Lethality
75. SMITH, Kendric C.
Stanford University
5 R01 CA 33738-04
Ionizing Radiation Mutagenesis
in E. coli
76. STEVENS, Reggie H.
University of Iowa
5 R01 CA 30967-03
Late Effects of In Utero
Iodine-131 Exposure
77. SUTHERLAND, Betsy M.
Associated Univ./Brookhaven Natl. Lab.
2 R01 CA23096-09
DNA Repair: Human E. coli
Photoreactivating Enzymes
78. SUTHERLAND, Betsy M.
Associated Univ./Brookhaven Natl. Lab.
5 R01 CA 26492-07
UV Transformation, DNA Repair
in Human Cells and Skin
79. THORNE, Michael
Intl. Com. on Rad. Protection
5 R01 CA 30163-05
Recommendations on Radiation
Protection
80. TODD, Paul W.
Pennsylvania State University
5 R01 CA 35370-03
Human Somatic Cell Mutagenesis
by Gamma Rays
81. ULLRICH, Robert L.
Oak Ridge National Laboratory
5 R01 CA 27531-05
Carcinogenic Interactions of
Radiation and Chemicals
82. ULLRICH, Robert L.
Martin Marietta Energy Systems
7 R01 CA 43322-01
Carcinogenic Interactions of
Radiation and Chemicals

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| 83. VERMA, Surendra
New England Medical Center, Inc.
5 R01 CA 36195-03 | Membrane Composition and Radiation
Damage |
| 84. WALDREN, Charles A.
Eleanor Roosevelt Inst. for Cancer Res.
5 R01 CA 36447-03 | Cell Genetic Damage at Low
Doses and Dose Rates |
| 85. WALLACE, Susan S.
New York Medical College
5 R01 CA 33657-05 | Repair of DNA Damage Induced by
Ionizing Radiation |
| 86. WALLACE, Susan S.
New York Medical College
5 R01 CA 35580-03 | Ionizing Radiation and
Transposon Mobility |
| 87. WARD, John F.
Univ. of California, San Diego
5 R01 CA 26279-07 | Mechanisms in Shouldered
Survival Curves |
| 88. WEISS, Herbert
Memorial Sloan-Kettering Cancer Center
5 R01 CA 31677-04 | Mechanisms of Radiation Damage
in Cells |
| 89. WILLIAMS, Jerry R.
Johns Hopkins University
5 R01 CA 39543-03 | X-Ray Induction of Cellular
Hypermutability |
| 90. ZAIN, Sayeeda B.
University of Rochester
2 R01 CA 36432-03 | Oncogenes, Oncogene Products in
Radiation-Induced Tumors |

CONTRACTS ACTIVE DURING FY 86

- | <u>Investigator/Institution/
Contract Number</u> | <u>Title</u> |
|--|---|
| 91. BENJAMIN, Stephen A.
Food and Drug Administration
Y01-CP-50506 | Neoplasia in Beagles after
Irradiation During Development |
| 92. BURR, William W.
Department of Energy
Y01-CP-50512 | Evaluation of Devices and
Techniques to Determine Previous
Radiation Exposure |
| 93. CRONKITE, Eugene P.
Department of Energy
Y01-CO-00712 | Radiation Leukemia - Prediction
of Low Dose Effects |
| 94. FRITZ, Thomas E.
Department of Energy
Y01-CP-50503 | Late Effects of Protracted
Irradiation in Dogs |

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|--|---|
| 95. FRY, R. J. Michael
Department of Energy
Y01-CM-20113 | Cocarcinogenesis: Ionizing and
Ultraviolet Radiation |
| 96. HOFFMAN, F. Owen
Department of Energy
Y01-CP-60504 | Pasture Grass Interception and
Retention of Iodine-131 |
| 97. STEVENS, Walter
University of Utah
N01-CO-23917 | Assessment of Leukemia and Thyroid
Disease in Relation to Fallout
in Utah |
| 98. STORER, John B.
Department of Energy
Y01-CM-20112 | Extrapolation of Radiation Risk |
| 99. ULLRICH, Robert L.
Department of Energy
Y01-CM-20111 | Radiation-Induced Myelogenous
Leukemia |



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